Ectopic Lymphotoxin Expression in the Pancreas and in the Central Nervous System Predisposes to Autoimmunity

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DISSERTATION

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2012

Die vorliegende Arbeit wurde von der Mathematisch-naturwissenschaftlichen Fakultät der Universität Zürich auf Antrag von Prof. Dr. Adriano Aguzzi angenommen.

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Abbreviations

ADAM	a disintegrin and metalloprotease
AIP	autoimmune pancreatitis
APC	antigen presenting cell
APRIL	a proliferation-inducing ligand
BAFF	B-cell activating factor
CNS	central nervous system
CRD	cystein-rich domain
DR3	death receptor 3
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr-Virus
ECM	extracellular matrix
EDA	ectodermal dysplasin
EDAR	ectodermal dysplasin receptor
FACS	fluorescence-activated cell sorting
FADD	Fas associated death domain
FcγR	Fc γ receptor
FDC	follicular dendritic cell
GC	germinal center
GFAP	glial fibrillary acidic protein
HEV	high endothelial venule
HVEM	herpes virus entry mediator
iBALT	inducible bronchus-associated lymphoid tissue
ICAM-1	intracellular adhesion molecule 1
IDDM	diabetes mellitus type 1
IKK	inhibitor of κB kinase
IKKα	inhibitor of κB kinase-α
IκB	inhibitor of κB
JNK	Jun N-terminal kinase
Lck	leukocyte-specific protein tyrosine kinase
LIGHT	homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM on T cells

LT	lymphotoxin
LTβR	lymphotoxin beta-receptor
LF	lactoferrin
MadCAM-1	mucosal addressin cell adhesion molecule 1
MBP	myelin basic protein
MHC	major histocompatibility complex
mLN	mesenteric lymph node
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
MZ	marginal zone
NALT	nasal associated lymphoid tissue
NF-κB	nuclear factor-κB
NIK	nuclear factor-κB inducing kinase
NK	natural killer cells
PLP	myelin proteolipid protein
PP	Peyer's patch
PSTI	pancreatic secretory trypsin inhibitor
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor-κB ligand
RF	rheumatic factor
SLE	systemic lupus erythematosus
SLO	secondary lymphoid organ
sLTβR-Ig	soluble LTβR immunoglobulin fusion protein
THD	TNF homology domain
TIM	TRAF interacting motif
TLO	tertiary lymphoid organ
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFSF	TNF superfamily
TRADD	TNFR associated death domain
TRAF	TNFR associated factors
TWEAK	TNF-like weak inducer of apoptosis
VCAM-1	vascular cellular adhesion molecule-1
VEGI	vascular endothelial cell-growth inhibitor

Summary

Autoimmune diseases are the consequence of an inappropriate immune response directed against self-antigens and as such they can affect most organs of the body including liver, heart, the endocrine system, the musculoskeletal apparatus and the central nervous system (CNS). They commonly start off at a young age and last throughout life, often resulting in severe disability. Collectively, autoimmune diseases affect up to 6% of the population and represent the 3rd greatest health issue after heart diseases and cancer. Therefore, much research is directed towards understanding the mechanisms associated with the development and pathology of autoimmune diseases. Aetiologically relevant preclinical animal models, preferably with spontaneous disease initiation are indispensable for studying disease pathogenesis and discovering new treatment strategies for human autoimmune diseases.

The inflammatory cytokines LTα and LTβ (LT) have been implicated in the context of a variety of autoimmune diseases (e.g. rheumatoid arthritis, Sjögren's syndrome, multiple sclerosis). The mechanisms attributed to LT during autoimmune diseases stem from their ability to trigger the development of secondary lymphoid organs, their involvement in the formation of ectopic lymphoid structures and their capability to control aspects of the organization of lymphoid microenvironments. During the past 20 years several transgenic mouse models have been generated, some of them by our research group (AlbLTαβ, lckLTαβ). However, none of these models so far were shown to develop an organ specific or a systemic autoimmune disease.

In my thesis I have analyzed serum and pancreatic tissue samples from human autoimmune pancreatitis (AIP) patients and I concluded that patients suffering of AIP display high expression of LT and downstream targets such as homeostatic chemokines. Interestingly, mice with acinar-specific LT expression *Tg*(Ela1-LTa,b) that I describe in my thesis, display spontaneous development of autoimmunity, reminiscent of AIP, including anti-nuclear antibodies (ANA), auto-antibodies against pancreatic self-antigens and renal immune-complexes, causally linking pancreatic LTαβ expression with AIP. Furthermore, in this novel mouse model I assessed the contribution of monocyte and lymphocyte cell populations towards inflammation and autoimmunity. Absence of lymphocytes (Ela-1LTab/Rag1$^{-/-}$) abrogated AIP, while lack of pro-inflammatory monocytes (Ela-1LTab/Ccr2$^{-/-}$) decreased early pancreatic tissue damage but failed to prevent AIP. Current treatment options for human AIP are limited, mostly administering corticosteroids, which frequently results in relapses.

The *Tg*(Ela1-LTa,b) mouse model can also serve as a useful tool to test potential new therapies: I compared prednisolone treatment with inhibitors of the CD40L-CD40 or the $LT\alpha_1\beta_2$-LTβR-signalling pathway in *Tg*(Ela1-LTa,b) mice. Corticosteroid treatment reduced pancreatitis but did not hamper auto-antibody production. Inhibition of the CD40-CD40L pathway failed to reduce the extent of AIP in *Tg* mice, although a decrease in serum IgG levels confirmed treatment efficacy. In contrast, inhibition of LTβR-signalling dampened auto-antibody production, chemokine expression, renal immune-complex deposition and fully abrogated AIP. Thus, suppression of LTβR-signalling might become a viable alternative or combinatorial treatment option for AIP.

A controversial role is attributed to LT in autoimmune reactions and demyelination in the CNS. To elucidate the role of astrocyte derived LT induced cytokine production and tissue destruction in CNS autoimmunity, I have generated a transgenic mouse model with targeted overexpression of Ltα and β on astrocytes under the control of the glial fibrillary acid protein promoter (*GFAP-Lta,Ltb).* Transgenic mice developed early onset of astrogliosis concomitant with increased expression of lymphoid chemokines and adhesion molecules. EAE was induced in age matched transgenic and wild type animals to assess the contribution of LT to EAE pathogenesis. *Tg*(*GFAP-Lta,Ltb)* mice showed a significantly earlier disease onset, more severe disease manifestation and relapse compared to age matched wild type littermates. As a next step, I intercrossed *Tg*(*GFAP-Lta,Ltb*) with mice bearing myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic T-cells (2D2/GFAP-LT). These double transgenic mice developed spontaneous inflammatory demyelinating disease - mainly localized in the spinal cord and cerebellum - with a 100% incidence. The inflammatory infiltrates in the spinal cord resembled ectopic lymphoid follicles, mainly consisting of $CD4^+$ T-cells and $B220^+$ B-cells. We hypothesize that the spontaneous disease development resulted from the attraction of myelin autoimmune T-cells via the inflammatory milieu in the CNS created by LT overexpressing astrocytes. This is the first study to our knowledge that describes the role of local CNS immunity in attracting myelin specific T-cells, facilitating to study the initiation and pathogenesis of a complex spontaneous demyelinating autoimmune disease.

Zusammenfassung

Autoimmunerkrankungen sind die Folge fehlgeleiteter Immunantworten gegenüber Eiweissmolekülen des eigenen Körpers (sogenannte Selbstantigenen) und können als solche nahezu alle Körperorgane unter anderem Leber, Herz, endokrine System, Muskelapparat und das zentrale Nervensystem (ZNS) betreffen. Gewöhnlich treten sie bereits im jungen Lebensalter auf und halten ein Leben lang vor, was häufig zu schweren körperlichen Einschränkungen führt. Autoimmunerkrankungen betreffen bis zu 6% der Bevölkerung und bilden nach Herz- und Krebs-erkrankungen das drittgrößte Gesundheitsproblem. Aus diesem Grund werden große Bemühungen in der Forschung unternommen, um die zugrunde liegenden Mechanismen der Entstehung und Pathologie von Autoimmunerkrankungen zu verstehen. Relevante prä-klinische Tiermodelle, bevorzugt mit spontaner Krankheitsentstehung, sind unerlässlich zur Untersuchung der Krankheitsverläufe und zur Entwicklung neuer Therapieansätze für humane Autoimmunerkrankungen.

Die Entzündungsmediatoren LTα und LTβ konnten in Verbindung mit einer Vielzahl verschiedenster Autoimmunerkrankungen gebracht werden u.a. Rheumatoide Arthritis, Sjörgren's Syndrom und Multipler Sklerose. Die Rolle von Lymphotoxinen in Autoimmunerkrankungen beruht auf ihrer Eigenschaft die Entwicklung sekundärer lymphoider Organe zu induzieren, der Mitwirkung an der Enstehung ektoper lymphoider Strukturen und ihrer Fähigkeit verschiedene Aspekte der Organisation des lymphoiden Microenvironment zu beeinflussen. Innerhalb der letzten 20 Jahre wurden verschiedenste transgene Mausmodelle entwickelt, einige davon durch unsere Arbeitsgruppe. Jedoch zeigt keines der bisher generierten Modelle Anzeichen einer Organ-spezifischen oder einer systemischen Autoimmunerkrankung.

Im Rahmen meiner Doktorarbeit habe ich Serumproben und Gewebematerial von Patienten mit Autoimmunpankreatitis (AIP) untersucht und feststellen können, dass Patienten, die an AIP leiden, eine hohe Expression von LT and seinen Zielgenen wie zum Beispiel homöostatische Chemokine aufweisen. Mäuse mit einer Azinar-spezifischen LT Expression (Ela1-LTa,b), welche ich im Rahmen meiner Doktorarbeit beschreibe, zeigten eine spontane Autoimmunerkrankung mit Merkmalen von AIP wie anti-nukleäre Antikörper, Autoantikörper gegen Pankreas spezifische Selbstantigene und renale Immunkomplexe. Dies deutet auf einen kausalen Zusammenhang zwischen der pankreatischen LT Expression und

der Enstehung von AIP hin. Zusätzlich untersuchte ich mit Hilfe dieses neuen Mausmodels die Rolle von Monozyten und Lymphozyten in Entzündung und Autoimmunität. Das Fehlen von Lymphozyten verhinderte die Enstehung einer AIP vollständig während in der Abwesenheit pro-inflammatorischer Monozyten nur eine Abschwächung der frühen pankreatischen Gewebeschädigung erfolgte jedoch keine Verhinderung der AIP möglich war. Derzeit sind die Behandlungsmethoden für humane AIP Patienten stark begrenzt und in den meisten Fällen erfolgt eine Behandlung mit Corticosteroiden, welche häufig zu Rückfällen führt. Dieses Mausmodel kann als Hilfsmittel zur Entwicklung neuer Therapieformen dienen: Zu diesem Zweck habe ich im *Tg*(Ela1-LTa,b) Mausmodel die Behandlung mit Prednisolon, mit Inhibitoren des CD40L-CD40 sowie des $LT\alpha_1\beta_2$-LTβR Signalweges verglichen. Eine Behandlung mit Corticosteroiden reduzierte die Entzündung, nicht jedoch die Produktion von Autoantikörpern. Die Inhibition des CD40L-CD40 Signalweges konnte zwar nicht den Grad der Gewebeentzündung senken, jedoch konnte über die Absenkung des Serum IgG Spiegels die Effektivität der Behandlung nachgewiesen werden. Im Gegensatz dazu resultierte die Inhibition des LTβR Signalweges in einer Verringerung der Autoantikörper und Chemokinproduktion, der Ablagerung renaler Immunkomplexe und führte zu einer vollständigen Unterdrückung der Gewebeentzündung. Die Inhibition des LTβR könnte daher eine neue Alternative oder Ergänzung in der Behandlung von AIP darstellen.

Die Rolle von Lymphotoxinen in Autoimmunprozessen und Demyelinierung in ZNS wird kontrovers diskutiert. Um die Rolle Lymphotoxin induziert Zytokinproduktion und Gewebschädigung in Astrozyten in Autoimmunerkrankungen des zentralen Nervensystems (ZNS) genauer zu untersuchen, habe ich ein transgenes Mausmodel mit gewebespezifischer Überexpression von LTα und β in Astrozyten unter der Kontrolle des glial fibrillary acid protein (GFAP) Promotors generiert. *Tg*(GFAP-LTa,LTb) Mäuse entwickelten eine frühe Astrogliose einhergehend mit der Expression lymphoider Chemokine und verschiedener Adhäsionsmolekülen. Um die Rolle von LT in der Pathogenese von EAE zu untersuchen wurde EAE in transgenen und Wildtyptieren gleichen Alters induziert. Im Vergleich zu den gleichaltrigen Kontrolltieren zeigten die transgenen Tiere ein signifikant früheres Auftreten der Erkrankung, eine schwerer Ausprägung der Erkrankung und Rückfälle nach Ausheilung der Erkrankung. Im nächsten Schritt wurden die transgenen Tiere mit Mäusen verpaart, welche einen transgenen T-Zell Rezeptor tragen, der spezifisch für das myelin-oligodendrocyte Glykoprotein ist. In doppelt transgenen Tieren entwickelte sich mit einer Inzidenz von 100% eine spontane, entzündliche, demyelinisierende Erkrankung, welche sich hauptsächlich auf Rückenmark und Kleinhirn begrenzte. Die entzündlichen Infiltrate im

Rückenmark ähneln ektopen Lymphfollikeln und bestehen hauptsächlich aus $CD4^+$ T-Zellen und $B220^+$ B-Zellen. Wir vermuten, dass die spontane Erkrankung auf die Rekrutierung der autoreaktiven T-Zellen in das ZNS aufgrund des inflammatorischen Milieus zurückgeht, welches durch die Überexpression von LT in Astrozyten verursacht wird. Dies ist nach unserem Wissen die erste Studie welche eine Rolle des lokalen ZNS Immunsystems in der Rekrutierung Myelin-spezifischer T-Zellen beschreibt und somit die Analyse der Entstehung und Pathogenese einer komplexen demyelinisierenden Autoimmunerkrankung ermöglicht.

1. Introduction

1.1 Autoimmunity

Autoimmunity is the failure of an organism to recognize its own proteins as *self*, which can consequently trigger an immune response against the own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease. Autoimmune diseases affect approximately 6% of the population and therefore they are the third largest disease burden after heart disease and cancer (Siatskas et al., 2006). Autoimmune diseases can affect most organs of the body including liver, heart, endocrine system, the musculoskeletal apparatus and the central nervous system (CNS). Autoimmune diseases can be broadly divided into organ-specific and systemic autoimmune diseases. Systemic autoimmune diseases include diseases such as systemic lupus erythematosus (SLE), scleroderma and polymyositis. The feature of these diseases is that the targeted antigens are located throughout the body. As the name implies, organ-specific autoimmune diseases involve specific organs of the body in which the target auto-antigen is found. Prominent examples of organ-specific autoimmune diseases include type 1 diabetes (insulin secreting cells of the pancreas), thyroiditis (thyroid), multiple sclerosis (myelin sheath of neurons), and as a recently accepted entity autoimmune pancreatitis (exocrine pancreas). They commonly start at a young age and then last throughout life, often resulting in severe disability (Pollinger et al., 2009).

Autoimmune diseases may arise by different mechanisms; genetic factors are important, but are not the only explanation for development of an autoimmune disorder. Its risk is also affected by environmental factors and by infections as well (Doan, Melwold et al., Immunology). The factors triggering the onset and modulating the course of the autoimmune diseases have remained obscure, a deficit of knowledge which sets limits to the design of specific and efficient therapies (Pollinger et al., 2009). Cytokines play a pivotal role in the pathogenesis of autoimmune diseases. The precise triggers for the breakdown of self-tolerance and the subsequent events leading to the induction of pathogenic autoimmune responses remain to be defined for most of the naturally occurring autoimmune diseases. Studies conducted in experimental models of human autoimmune diseases and observations in patients have revealed a general scheme in which pro-inflammatory cytokines contribute to

the initiation and propagation of autoimmune inflammation, whereas anti-inflammatory cytokines facilitate the regression of inflammation and recovery from acute phase of the disease (Moudgil and Choubey, 2011). Until recently, the pathogenesis of autoimmune diseases was examined and analysed largely in the context of the T helper 1 and 2 (Th1/Th2) cytokine balance, with the two T-cell subsets mutually cross-regulating each other (Abbas et al., 1996) (Mosmann et al., 1986) (Coffman, 2006). Interestingly, over the past decade, the interleukin (IL)-17/IL-23 axis has rapidly emerged as the new paradigm, attributing an important role for IL-17 in the development of various autoimmune diseases such as RA (Lubberts et al., 2001) and MS (Cua et al., 2003) (Komiyama et al., 2006).

To date, however, it is still elusive how auto-antigens drive the pathogenesis of autoimmune disease, particularly how they interact with the triggers that initiate inflammation and hence, the clinical onset of disease. Animal models are indispensable for studying disease pathogenesis and discovering new treatments for human organ-specific autoimmune diseases, and developing better diagnostic tools (Wekerle et al., 2012). Although animal models make it possible to identify and manipulate certain autoimmune pathogenic mechanisms, they are limited by other factors.

First, the mode of disease initiation is usually highly artificial. This is particularly true for the models of MS and RA, which are usually induced by active immunization with an auto-antigen emulsified in aggressive immune adjuvants (Billiau and Matthys, 2001) or by transfer of *in vitro*–activated autoimmune lymphocytes (Ben-Nun et al., 1981). Second, the treatment in these therapy studies is often administered very early in the course of the induced autoimmune disease and is even sometimes given as a prophylactic, in which case it precedes the onset of clinical disease. In contrast, any therapy given to patients with autoimmune disorders is usually administered to counter a mature, often chronic disease (Wekerle et al., 2012). In my thesis I will describe two novel transgenic mouse models, to elucidate the role of the pro-inflammatory cytokines, Lymphotoxin (LTα and LTβ) in the course of autoimmune pancreatitis (AIP) and an autoimmune disease of the central nervous system (CNS).

1.2 The Lymphotoxin signalling

1.2.1. The TNF superfamily – receptor and ligand interactions

The Lymphotoxin β receptor (LTβR) signalling axis belongs to a complex gene family of cytokines and their corresponding receptors, which is called the tumor necrosis factor (TNF) –

superfamily (TNFSF). At present, TNFSF is composed of 19 different ligands, including, for example, TRAIL, RANKL, BAFF, and CD40. The ligands mediate their cellular response through 29 type I transmembrane receptors, which contain an extracellular cysteine-rich domain and in some cases (for example, TNFR1, TRAIL R1, TRAIL R2, Fas) a death domain (**Figure 1**) (Schneider et al., 2004) (Aggarwal, 2003). Activation of the TNFR members via their ligands affects cell proliferation, survival, differentiation and apoptosis of responding cells.

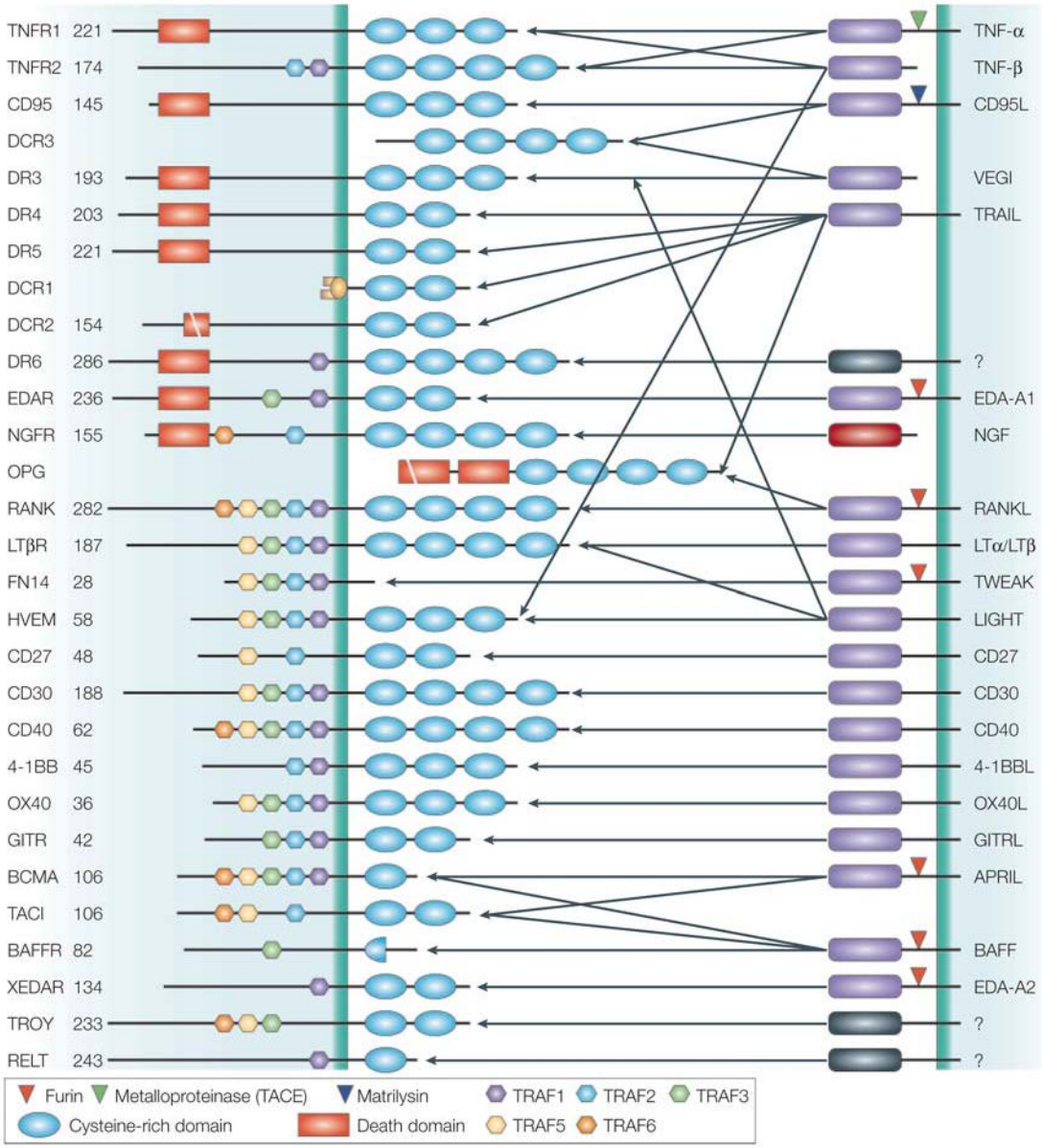

Figure 1. Interactions between TNF superfamily ligands and their known receptors. Ligands and receptors are shown in a diagrammatic form. Many ligands bind more than one receptor indicated with arrows. Ligands for RELT, TROY and DR6 have not been discovered yet. A common motif of all TNF-superfamily receptors is the cysteine-rich domain in the extracellular portion (blue circles). Red boxes in the cytoplasmic part of the receptors indicate the presence of a death domain. (Adapted from Aggarwal et al., 2003)

Except for lymphotoxin alpha (LTα) and vascular endothelial cell-growth inhibitor (VEGI), which are secreted, all TNFSF ligands are type II transmembrane proteins (intracellular N-terminus) which are biologically active as self-assembling, non-covalently bound trimers (Hehlgans and Pfeffer, 2005). In the membrane bound form they can initiate signalling by activating cell surface receptors on adjacent cells (juxtacrine signalling) or on the producing cell (autocrine signalling). The soluble ligands consequently can act also on distant cells. The C-terminal extracellular domain contains a TNF homology domain (THD). This trimeric domain is responsible for receptor binding and its amino acid homology between family members is 20-30%. The THD is present in the form of an antiparallel β-sheet that allows the ligands to assemble trimeric tertiary structure with three exposed receptor-binding sites (Bodmer et al., 2002) (Aggarwal, 2003). Although these ligands are generally synthesized as membrane bound proteins, soluble forms can be released from the cell surface by limited proteolysis. Distinct proteases are involved in this process, depending on the ligand: TNF and receptor activator of nuclear factor-κB ligand (RANKL) are cleaved by members of the a disintegrin and metalloprotease (ADAM) family of metalloproteases (Black et al., 1997) (Lum et al., 1999); matrilysin acts on Fas ligand (FasL) (Powell et al., 1999); and B-cell activating factor (BAFF), ectodermal dysplasin (EDA), TNF-like weak inducer of apoptosis (TWEAK) and a proliferation-inducing ligand (APRIL) is cleaved by proteases of the furin family (Schneider et al., 2004).

Most of the TNF receptor family members are type I transmembrane proteins. Their hallmark is a cystein-rich domain (CRD) in the extracellular portion. The CRDs are pseudo-repeats typically containing six cysteine residues engaged in the formation of three disulfide bonds (Bodmer et al., 2002). The number of CRDs can range from one partial motif (BAFF-receptor) up to 6 found in CD30 (Aggarwal, 2003). Based on their intracellular sequences the members of the TNFR superfamily can be classified into three major groups.

1. The first group, including FAS (CD95), TNFRI, TNF related apoptosis inducing ligand receptor 1 (TRAIL-R1) (DR4), TRAIL-R2 (DR5), TRAIL-R4 (DcR2), TNF-like receptor apoptosis mediating protein (TRAMP) (DR3), Ectodysplasin (EDA) receptor (EDAR) contains so called death domains (DD) in their cytoplasmic domains.

Binding of TNF superfamily ligands to their DD containing receptors mediate signalling through intracellular death domain containing adaptors, such as FAS-associated death domain (FADD) and TNFR associated death domain (TRADD). These molecules activate the caspase cascade and subsequently induce apoptosis (Dempsey et al., 2003).

2. The second group of receptors, including TNFR-II, CD27, CD 30, CD40, LTβR, OX40, 4-1BB, BAFFR, BCMA, RANK, GITR, TACI, Fn14, HVEM, RELT, XEDAR and TROY contain one or more TNF-receptor associated factor (TRAF)- interacting motifs (TIMs) in their cytoplasmic domain. Activation of TIM containing TNFR family members leads to the recruitment of TRAF family members and the subsequent activation of downstream signal pathways like nuclear-factor κB (NF-κB) and Jun N-terminal kinase (JNK) pathways, p38, extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K) (Hehlgans 2005).

3. The third group of receptors including TRAIL-R3 (DcR1), DcR3 and osteoprotegerin (OPG) does not contain intracellular signalling domains or motifs. This group of receptors can effectively compete with the other two groups of receptors for their corresponding ligands. These decoy receptors therefore function by impeding the activation of signal transduction pathways of other TNF receptors (Dempsey et al., 2003).

The majority of the TNF superfamily ligands are expressed by cells of the immune system, including B-cells, T-cells, NK cells, monocytes and dendritic cells. The only exception is VEGI, which is expressed mainly by endothelial cells. However, the TNF receptors are expressed by a wide variety of cells that have both hematopoietic and non-hematopoietic origins. Thus, numerous activities are assigned to TNF superfamily members, in particular, their profound role in regulation of both normal and pathogenic immune responses (Grewal, 2009).

1.2.2. The history of LT and TNF – the prototypic members of the TNF superfamily

In the 1960s and early 1970s, two distinct molecules were described based on their ability to promote cell death in target cells (for example, murine fibrosarcoma L-929 cells L cells) (Ruddle and Waksman, 1967) (Granger and Williams, 1968). In 1967, Ruddle and colleges first described a “cytotoxic factor” in an experiment where isolated lymph node cells from rats showed toxicity on fibroblasts in the presence of specific antigens. One year later in 1968, a molecule with cytotoxic activity produced by lymphocytes was reported and named lymphotoxin (LT) (Granger and Williams, 1968) (Granger et al., 1969). In 1975, a substance was depicted causing efficient necrosis in various tumor types in vivo throughout a cytotoxic activity and was thus denominated ‘tumor necrosis factor’ (TNF) (Carswell et al., 1975). These molecules subsequently caught attention of several research groups using these

cytotoxic, tumor necrotizing capabilities for their therapeutic potential in the treatment of human cancer. In 1984, human LT was the first cytokine to be purified from a B-lymphoblastoid cell line (Aggarwal et al., 1984) and thereafter its amino acid sequence and structure was determined (Aggarwal et al., 1985) (Nedwin et al., 1985a). Neutralization of LT activity by LT-specific antibodies led to the isolation of a second cytotoxic factor from a human myeloid cell line, already known as TNF. The determination of the amino acid sequence of TNF revealed its close relationship to LT, leading to a renaming of LT into TNFβ. Due to the discovery of similarities in amino acid sequence of LT and TNF, and cloning their complementary DNA, it eventually became evident that these two cytokines are the prototypic members of the TNF superfamily (TNFSF) (Hehlgans and Pfeffer, 2005). Genes encoding these two TNF proteins have been cloned from mouse and man, and in both cases they are found in close proximity to each other (in mouse on chromosome 17 (Nedospasov et al., 1986) in man on the short arm of chromosome 6 (Nedwin et al., 1985b)) within the major histocompatibility complex (MHC) region. Roughly a decade later a third ligand was found in the same locus and named lymphotoxin β (LTβ, TNFSF3) (Pokholok et al., 1995) while subsequently TNFβ was termed LTα or TNFSF1B (tumor necrosis factor super family member 1B).

1.2.3. Signalling in the core TNF family

Under physiological conditions, LTα and LTβ are expressed by activated lymphocytes, a subset of resting B-cells, natural killer (NK) - and lymphoid tissue-inducer (LTi) cells. The LTβR is expressed mainly by non-haematopoietic and myeloid cells (Gommerman and Browning, 2003), thus facilitating a crosstalk between immune cells and the stroma. Although LTα mRNA expression was shown to be inducible in B-cells, LTβ mRNA is constitutively produced (Browning et al., 1993) (Worm and Geha, 1994).

The immediate TNF family is defined by four closely related ligands, which include LTα, LTβ, TNF and LIGHT and their cognate receptors TNFR1, TNFR2, LTβR and HVEM (**Figure 2**). LTα and LTβ can form 3 different ligands (Schneider et al., 2004). As secreted homotrimeric molecule, LT (LTα3), like TNF, signals via TNFR1 (p55), TNFR2 (p75), and herpes virus entry mediator (HVEM). Unlike LTα, LTβ has not been detected in a secreted form, rather as a type II transmembrane protein which binds LTα to form 2 distinct membrane anchored heterotrimers: $LT\alpha_1\beta_2$ (predominant form) and $LT\alpha_2\beta_1$ (Browning et al.,

1995). $LT\alpha_1\beta_2$ triggers LTβR, whereas $LT\alpha_2\beta_1$ was reported to bind TNFR1 and TNFR2, but this is a minor form expressed by T-cells (< 2%) with an undefined biological role (Ware, 2005). LIGHT (TNFSF14), the most recently described member is an alternative ligand for the LTβR but also interacts with HVEM (Schneider et al., 2004).

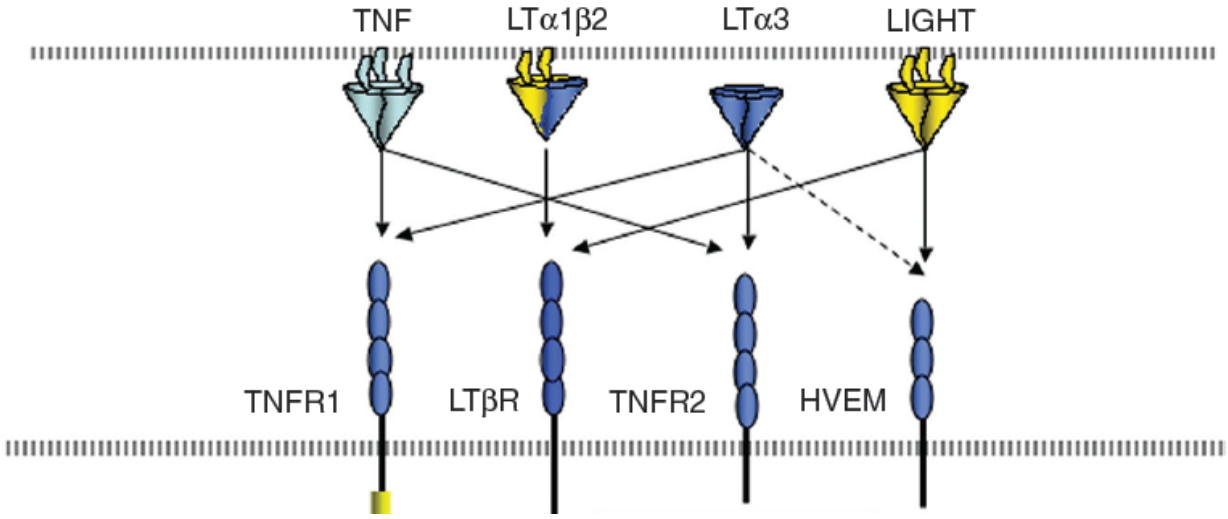

Figure 2. Ligand and receptor interactions of the tumor necrosis factor core family. Solid lines indicate high affinity interactions; dashed line refers to weak interactions. Two fundamental pathways can be defined. The TNF pathways are activated by TNF or LTα homotrimer (LTα3), signalling through the two TNF receptors (TNFR1, TNFR2). The LTβR signalling is triggered by two ligands, the LTαβ heterotrimer (LTα1β2) and homotrimeric LIGHT (adapted from Ware et al 2005).

TNFR and LTβR-signalling pathways lead to the activation of NF-κB through different mechanisms (**Figure 3**). The NF-κB signalling is a major regulator of innate and adaptive immune responses, cell survival or apoptosis, cellular stress responses, development and maintenance of lymphoid organs. In mammals, the NF-κB family consists of five structurally conserved members: NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), Rel A (p65), Rel B and c-Rel. These NF-κB proteins can form different homo- and heterodimeric complexes and are tightly controlled by several inhibitory proteins, belonging mostly to the IκBα family (Bonizzi and Karin, 2004). So far, two different signalling pathways are characterized: the canonical (classical) and the non-canonical (alternative) pathway (Dejardin et al., 2002).

Activation of the canonical NF-κB pathway mainly occurs in response to inflammatory cytokines such as TNF, and IL-1, engagement of the T-cell receptor and in response to bacterial infection (for example, lipopolysaccharide). NF-κB1 is kept in the cytosol by inhibitors such as IκB (inhibitor of κB). Upon a wide variety of stimuli the IKK (IκB kinase)

complex (IKKα, IKKβ and the regulatory subunit IKKγ, also called NEMO) gets phosphorylated, which results in the ubiquitination and degradation of IκB by the proteasome allowing the NF-κB1/RelA heterodimer to translocate in the nucleus.

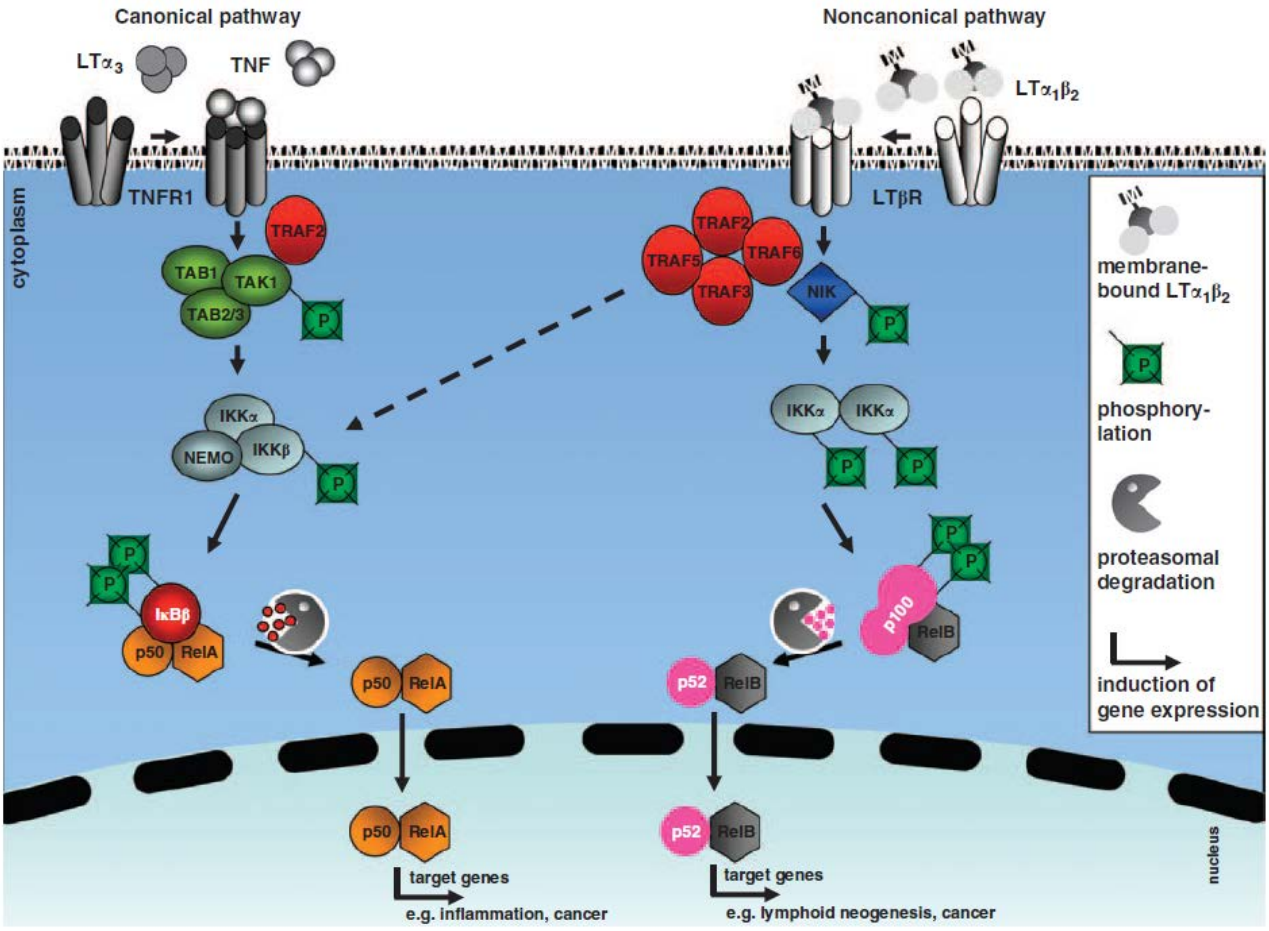

Figure 3. TNFR and LTβR signalling pathways lead to the activation of NF-κB through different pathways. (Left) The canonical pathway of NF-κB activation is induced by a large number of agonists, representatively, TNF and LTα3 are shown. Stimulation of the TNF receptor (TNFR) leads to the activation of the TAK1 complex through TRAF proteins. This leads to the activation of the IKK complex by phosphorylation of NEMO/IKKγ and IKKβ, which results in the phosphorylation of IkBα, its subsequent ubiquitylation and proteasomal degradation. Finally, the heterodimeric p50-RelA complex translocates to the nucleus, where it binds to specific kB-sequences and induces the expression of target genes, including pro-inflammatory cytokines or genes involved in cell survival, cell proliferation and pro-carcinogenesis.

(Right) The non-canonical pathway becomes activated by membrane-bound LTα1β2 heterotrimers, which results in the activation of NF-κB-inducing kinase (NIK). This leads to the phosphorylation and activation of the homodimeric IKKα complex. As a consequence, the precursor p100 becomes phosphorylated and proteasomally processed to the p52 subunit establishing now the RelB-p52 heterodimeric complex that translocates to the nucleus and induces the expression of target genes. Besides, LTβR was shown to activate the canonical NF-κB signalling pathway (Adapted from Wolf et al. Oncogene).

Activation of the classical NF-κB pathway predominantly induces expression of inflammatory genes, such as *VCAM1*, *CCL14 (MIP-1β)* and *CXCL2 (MIP-2)* (Dejardin et al., 2002) (Schneider et al., 2004). Interestingly, LTβR activation (through LIGHT or LTαβ heterotrimers) can also activate the canonical NF-kB (Dejardin et al., 2002) (Haybaeck et al., 2009).

Stimulation of a subset of TNFR family members, such as LTβR and CD40, can activate the non-canonical NF-κB pathway (Coope et al., 2002) (Pomerantz and Baltimore, 2002). These stimuli appear to converge upon NF-κB -inducing kinase (NIK) (Xiao et al., 2001), which is required for IKKα phosphorylation (Ling et al., 1998). In this pathway, IKKα is thought to exist as a homodimer, independent of NEMO/IKKγ and IKKβ (Senftleben et al., 2001). Activated IKKα phosphorylates p100, thereby inducing its proteasome dependent processing to p52. The p52/RelB dimer then translocates into the nucleus to induce gene expression through NF-κB activation by the $LT\alpha_1\beta_2$, LTβR, NIK, IKKα pathway. This pathway is crucial for development and maintenance of spleen architecture, follicular dendritic cell maturation, lymph node, Peyer's patch and NALT organogenesis as well as regulation of B-cell survival (Weih and Caamano, 2003) (Wolf et al., 2010). The alternative pathway activates genes implicated in secondary lymphoid organogenesis and homeostasis such as *CCL21 (SLC), CCL19 (ELC), CXCL13 (BLC), CXCL12* (*SDF-1*) and *BAFF* (Dejardin et al., 2002).

1.3 Lymphotoxin in health and disease

1.3.1. The biological functions of LT

Initially, LTα and TNFα were thought to play similar biological functions. However, targeted gene deletion of several members (LTα, LTβ, LTβR, TNFR1, TNFR2 and TNFα) of the immediate TNF family altered this view, and allowed the dissection of various biological functions attributed to LT.

1.3.2. LTβR-signalling and lymph node organogenesis

The first evidence that LT is involved in the development of lymphatic tissues was provided by generation of a mice model lacking LTα (De Togni et al., 1994; Liepinsh et al., 2006).

$LT\alpha^{-/-}$ mice (which are deficient in membrane bound $LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$ heterotrimers and $LT\alpha$ homotrimer) are devoid of all peripheral and mesenteric lymph nodes (mLNs) as well as Peyer's patches (PPs). Of note, it has been reported that certain number of $LT\alpha^{-/-}$ mice develops lymph node like mesenteric structures (1-3 % according to (Fu et al., 1997) and up to 20% according (Banks et al., 1995)). Since LT-deficient mice produce normal levels of TNFα, it is apparent that this function in lymphoid organogenesis is peculiar to LT (Kratz et al., 1996).

To elucidate the distinct role of LTα and LTβ in the lymphoid organogenesis a mice deficient in LTβ expression were generated (Alimzhanov et al., 1997) (Koni et al., 1997). $LT\beta^{-/-}$ mice, devoid of heterotrimeric $LT\alpha_1\beta_2$ (and $LT\alpha_2\beta_1$) that binds LTβR, but continue to express LTα, exhibit a similar phenotype to $LT\alpha^{-/-}$ mice except that the cervical (cLNs) and mLNs are retained (Ngo et al., 1999). This data suggested that LTα can function independently from LTβ during lymphoid organogenesis (Wolf et al., 2011).

Generation of $LT\beta R^{-/-}$ mice enabled to define the role of LTβR in lymphoid development, micro-architectural organization and lymphoid neogenesis; and to dissect whether these processes require heterotrimeric LT (via LTβR) or $LT\alpha_3$ homotrimer (via TNFR1 or TNFR2). $LT\beta R^{-/-}$ mice have a phenotype very similar to LTα deficient animals concerning splenic microarchitecture. In contrast to LTα and LTβ deficient mice histological studies revealed that mLNs, cLNs and inguinal lymph nodes as well as PPs are completely undetectable in $Lt\beta R^{-/-}$ mice, and at the putative locations of LNs, cavernous spaces with endothelial linings could be found, indicating that LN development is completely arrested (Futterer et al., 1998). Thus it can be concluded that LTβR-signalling is crucial for the organogenesis of the peripheral LN system.

Notably, similar to $LT\alpha^{-/-}$ mice, $LT\beta R^{-/-}$ mice show inflammation in the liver, lung, pancreas, and kidney mainly composed of activated lymphocytes. It is believed that this possibly reflects a state of autoimmunity or an in vivo situation in which immune cells have started to colonize new niches in non-lymphoid organs due to the lack of various secondary lymphoid organs (Futterer et al., 1998) (Chin et al., 2003). The generation of LTβR-Ig, a fusion decoy protein which enables a functional inhibition of LTβR-signalling, helped to study the role of surface LTαβ complexes in the development of peripheral lymphoid organs. By varying the gestational day of LTβR-Ig injection (by specifically blocking signalling through LTβR), the genesis of LNs and PPs appeared to proceed in an anteriorto-posterior order (mesenterics, brachials, axillaries, inguinals, and poplietals, PPs) (Rennert et al., 1996). Postgestational

LTβR-signalling during the first 6 weeks after birth is shown to be critical for the development of intestinal lamina propria B cells and isolated lymphoid follicles of the intestine (Lorenz et al., 2003).

1.3.3. LTβR-signalling and splenic micro-architecture

Both LTα$^{-/-}$ and LTβR$^{-/-}$ mice lack an organized splenic microarchitecture, germinal centers (GC), follicular dendritic cell (FDC) networks from the B-cell follicles, compartmentalization into T- and B-cell areas, display size reduction of white pulp follicles and are devoid of a distinct marginal zone (MZ) (Wolf et al., 2011) (De Togni et al., 1994). Of note, in LTβ$^{-/-}$ mice splenic architecture is somewhat less disturbed than in LTα$^{-/-}$ mice (Ngo et al., 1999). To dissect cell type-specific LT contribution in the complex LT-deficient phenotype, LTβ deficient mice were generated by conditional gene targeting (Tumanov et al., 2003). The obtained results imply that LT complex expressed by B-cells plays a major role in the maintenance of spleen structure, while surface LT expressed by T-cells provides a complementary but distinct signal.

The spleens of LTα$^{-/-}$ and LTβ$^{-/-}$ mice exhibit a dramatic reduction in the expression of homeostatic chemokines (CCL19, CCL21, and CXCL13) (Ngo J.exp med. 1999). In the spleen CXCL13 (or B lymphocyte chemoattractant) is primarily expressed by FDCs to attract B-cells into the splenic follicle. CXCL13 signals through its cognate receptor CXCR5 on the surface of B-cells, resulting in sustained LTαβ expression (McCarthy et al., 2006). LTβR-signalling also controls the level of CCL19 and CCL21, chemokines that serve to attract T-cells and dendritic cells (DCs) (Ngo et al., 1999). Likewise, CXCL13 maintains the positioning of B-cells in the follicle, whereas CCL19/CCL21 draws T-cells into the T-cell zone. Thus, the mechanism whereby LTβR-signalling controls lymphoid tissue development is at least partially explained by the deregulation of the expression of homeostatic chemokines such as CXCL13, CCL19, and CCL21, which recruit immune cells to the developing lymphoid anlage (Mebius, 2003) (Muller and Lipp, 2003). In addition to lymphocyte organization in white pulp of the spleen, LTβR-signalling also influences the marginal zone. The marginal zone separates the red and white pulp regions of the spleen and it is important for sampling antigens from the blood. Expression of adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and mucosal

addressin cell adhesion molecule 1 (MAdCAM-1) is induced by LTβR-signalling, and they play an important role in retaining MZ-resident cells (McCarthy et al., 2006).

1.3.4. The role of Lymphotoxin in regulation of the immune responses

Humoral immune responses result in the migration of a subset of antigen specific B-cells into the germinal center, within the B-cell follicles of secondary lymphoid tissues. GCs are specialized microenvironments where antigen-specific B-cells proliferate, undergo class switch, somatic hypermutuation and affinity maturation in order to generate high affinity antibodies. FDCs form tight networks within the GC where they provide survival factors to rapidly dividing centroblast B-cells and present antigen-antibody complexes to B-cells (McCarthy et al., 2006) (Park and Choi, 2005). In order to study the contribution of LT in mounting of a proper immune response, $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $LT\beta R^{-/-}$ mice were immunized. A normal IgM response was observed in all mice, but only minimal amounts of IgG production was measured, which indicates a defect in class switch (Koni et al., 1997) (Futterer et al., 1998). In LTβ deficient mice, which lack all FDC networks, GC formation is diminished but they do form, furthermore high-affinity antibody production is not completely absent but is delayed by a few weeks (Koni and Flavell, 1999). In addition, affinity maturation leading to high-affinity antibodies is impaired in LTβR deficient mice (Futterer et al., 1998). Recently, somatic hypermutation was shown to occur outside the germinal centers in extrafollicular compartments, perhaps explaining the observations in LT-deficient animals (Gommerman and Browning, 2003) (William et al., 2002). Reconstitution experiments in which wild type mice received primed splenocytes from $LT\alpha^{-/-}$ mice, Fu and colleagues demonstrated a substantial impairment in the formation of memory B-cells in $LT\alpha^{-/-}$ mice (Fu et al., 2000). Of interest, functional memory T-cells could be recovered from $LT\alpha^{-/-}$ donors. As previously mentioned mice deficient in LTα, LTβ or LTβR are born with impairment of LN and PPs biogenesis and disturbed splenic microarchitecture. Most likely these structural disturbances are associated with impaired high affinity isotype-switched Ig responses following immunization (Fu et al., 2000). Further, an important role of LTβR-signalling was recently shown in dendritic cell function (Summers-DeLuca et al., 2007). The absence of LTαβ expression on helper T-cells results in compromised T-cell priming by DCs ex vivo, and $LT\beta^{-/-}$ $CD4^+$ T-cell responses were impaired in vivo. This shows that $CD4^+$ T-cell derived LT is a critical participant in mediating full DC maturation and stimulatory function.

1.3.5. The role of Lymphotoxin in pathological processes

Members of the LT/TNF family can not only contribute to the development and maintenance of secondary lymphoid organs, but they also play an important role in inflammatory processes. In the course of chronic inflammation or autoimmune disease new lymphoid tissues, so called tertiary lymphoid organs (TLOs) develop ectopically in non-lymphoid organs (Ruddle, 1999). These structures mimic secondary lymphoid organs (SLOs) anatomically, and also share functional characteristics, such as germinal center reactions. Further, they are critical for B-cell isotype class switching and affinity maturation (Henry and Kendall, 2010). However, unlike lymph nodes, TLOs are not encapsulated, suggesting an intimate interaction with the inflamed tissues. The presence of TLOs is thought to allow antigen presentation at the local mucosal tissue with high efficiency and reduce the possibility of disseminating infection (Zhang and Lu, 2012). The process by which highly organized TLOs arise *de novo* during chronic inflammation has been referred to as lymphoid neogenesis (Kratz et al., 1996). For the induction of TLOs LT activates endothelial cells to express adhesion molecules (e.g., V-CAM, I-CAM) (Cuff et al., 1999) (Cuff et al., 1998) and regulates the induction of homeostatic and a variety of pro- and anti-inflammatory chemokines (e.g., CXCL13, CCL19, CCL21, CCL5, CCL2, CXCL10). These TLOs display cells or structures known to depend on LTβR-signalling (e.g., FDCs; high endothelial venules (HEVs) (Browning, 2008) (Drayton et al., 2006). HEVs are postcapillary vessels of secondary lymphoid organs composed of endothelial cells with a characteristic cuboidal morphology. Lymphocytes selectively adhere to and migrate across HEVs to initiate immune responses (Kawashima et al., 2009). Interestingly, HEVs were also observed in chronically inflamed non-lymphoid tissues (Drayton et al., 2006), and at the same time a direct role of LTβR-signalling as a requirement for the homeostatic control of HEV differentiation and function was described (Browning et al., 2005). A recent study shows that dendritic cells, which are well known for their role in antigen presentation to T lymphocytes, control the entry of naive lymphocytes to lymph nodes by modulating the phenotype of HEVs. The effect of DCs on HEV endothelial cells is direct and requires LTβR-dependent signalling (Moussion and Girard, 2011).

Several human inflammatory (e.g., hepatitis B and C virus-induced chronic hepatitis, *Helicobacter pylori*-induced gastric inflammation (Drayton et al., 2006) (Haybaeck et al., 2009) and autoimmune diseases (e.g. rheumatoid arthritis, Sjögren's syndrome, multiple

sclerosis (Hjelmervik et al., 2005) (Serafini et al., 2004) (Takemura et al., 2001) are associated with LT upregulation and TLO formation. It is likely that immune responses to self-antigens expand in these de novo lymphoid organs, as they allow co-localization of antigen specific T-and B-cells with antigen presenting cells (APCs) (Spahn et al., 2005). Various animal models were generated to recapitulate inflammation, accompanied by lymphoid neogenesis in the context of development of these autoimmune and inflammatory disorders. These models either utilize immunization with self-antigens, or transgenic expression of TNFSF members and its target genes (Summarized in **Table 1**).

Disease	Target tissue	TLO characteristic	References
Autoimmune diseases:			
Prediabetic NOD mice	pancreatic islets, salivary glands	Lymphoid aggregates with CCL21+ stromal cells and MADCAM1+ HEVs	(Faveeuw et al, 1994)
Collagen induced arthritis	Joints	B-cell follicles with GL7+ GCs	(Zheng B. et al. 2005)
Experimental autoimmune encephalomyelitis	CNS	Intrameningeal B-cell follicles with FDCs and GCs	(Magliozzi et al. 2004)
Ectopic expression			
RIP-TNF	pancretic islets, kidney	T cell–B cell compartments, HEVs (MAdCAM-1, PNAd), FDCs, DCs, plasma cells with IgG and IgM, CCL21, CXCL13	(Kratz. et al. 1996)
RIP-LTa, RIP-LTb	pancretic islets, kidney	T cell–B cell compartments, CCL19, CCL21, CXCL13 FDCs, DCs, HEVs (MAdCAM-1, PNAd)	(Drayton et al. 2003)
RIP-SLC (CCL21)	Pancreatic islets	T cell–B cell compartments, naive T cells, DCs, HEVs (MAdCAM-1, PNAd), FDCs	(Fan L. et al. 2000, Chen SC et al. 2002)
RIP-BLC (CXCL13)	Pancreatic islets	T cell–B cell compartments, DCs (no FDCs), HEVs (PNAd, HEC-6ST, MAdCAM-1) CCL21	(Bistrup A. et al. 2004)
RIP-ELC (CCL19)	Pancreatic islets	Small infiltrates, T cell–B cell compartments, HEVs (PNAd)	(Luther SA et al. 2002)
RIP-CXCL12	Pancreatic islets	Small infiltrates, mainly naive B cells, few T cells, DCs, plasma cells	(Luther SA et al. 2002)
Alb-LTab	Liver	B cells, FDCs, IgD+ and IgG1+ cells, DCs, PNA+ clusters	(Haybaeck et al. 2009)
Ela1-Lta,b	Exocrine pancreas	T cell–B cell compartments, FDCs, HEVs (PNAd), Germinal Centers (CD21/35)	(Seleznik et al.)
Tg-CCL21	Thyroid	T cell–B cell compartments, CD62L+ T cells, CD11c+ DCs, HEVs (PNAd)	(Martin, A.P. et al. 2004

Table 1. Mouse models of inflammation that are accompanied by lymphoid neogenesis (Adapted from Drayton et al., 2006 and Aloisi et al., 2006).

LTα or LTαβ expression under the control of the rat insulin promoter II (RIP) induces chemokine expression and follicular lymphocytic infiltrations ($CD4^+$, $CD8^+$ T-cells, $B220^+$ B-

cells, F4/80$^+$ macrophages, CD11c$^+$ dendritic cells) with GCs, HEVs and mature FDC networks in the kidney and pancreas (Picarella et al., 1992) (Kratz et al., 1996) (Drayton et al., 2003). Although insulitis was found in the case of many but not all pancreatic islets, RIPLTα mice did not progress to diabetes until the age of 8–12 months (Picarella et al., 1993). Notably, in RIPLTα kidneys pathological changes were observed, including mesangial proliferation and mesangiolysis within the glomeruli. Subsequent studies also demonstrated that overexpression of lymphoid homeostatic chemokines such as CCL21 or CXCL13 under the RIP promoter resulted in lymphotoxin dependent lymphoid neogenesis (Chen et al., 2002) (Luther et al., 2000)

Recently, - as a new paradigm in the TLO formation - IL-17, produced by T-cells was identified as being required for the formation of inducible bronchus-associated lymphoid tissue (iBALT), a type of TLO that forms in the lungs during chronic inflammation and infection (Rangel-Moreno et al., 2011). Using various knockout mice, the authors also showed that LTα and homeostatic chemokines, such as CXCL13, CCL19 and CCL21a, were required for the further organization of iBALT. Blockade of IL-17 reduced the number and size of iBALTs but did not affect their architecture. Thus, IL-17 is proposed to be important in TLO induction, while the maintenance and structural organization of iBALTs is LT dependent (Zhang and Lu, 2012). Of note, a recent study showed the generation of iBALT in IL-17 knock out animals, claiming that the general conclusion reached by Rangel-Moreno *et al*. that iBALT formation depends on IL-17 is inappropriate (Fleige et al., 2012).

Overexpression of LTαβ under the liver-specific albumin promoter (AlbLTαβ) led to chronic hepatitis in transgenic mice with an incidence of 100% at the age of 9 month. The pathological changes in this mouse model include organized portal and lobular lymphocytic infiltrates, proliferating hepatocytes and oval cells, presence of FDCs and GCs leading to mild, chronic liver damage. Interestingly, in approximately one-third of the mice at an age of >12 months (hepatocellular carcinoma) HCC can be detected (Heikenwalder et al., 2005) (Haybaeck et al., 2009).

Mice expressing LTα and LTβ under the control of the distal leukocyte-specific protein tyrosine kinase (lck) promoter were generated, resulting in LT expression on developing thymocytes within the thymus and on peripheral T cells in various lymphoid organs (Heikenwalder et al., 2008). In this case transgene expression on thymocytes led to their activation accelerated thymic involution, aberrant T cell development, and an altered thymic microarchitecture. Thymic involution, caused by massive apoptosis of thymic epithelial cells, was rescued by the removal of either TNFR1 or LTβR in the stromal compartment, but not by

depletion of overall TNFR2, LTα, or LTβ expression. Therefore, ectopic thymic LTαβ expression induces toxicity on thymic stromal cells, which is mediated by two non-redundant pathways: LTα3 signalling through TNFR1 and LTα1β2 signalling through LTβR (Wolf et al., 2011).

1.3.6. Therapeutic potential of modulating LTβR signalling in inflammatory and autoimmune diseases

As mentioned, LTβR is a TNFSF member best known for its role in lymphoid organogenesis and maintenance of lymphoid tissue organization. However, in addition to the well described function, a number of reports identify the involvement of LTβR-signalling in inflammatory processes, infectious diseases and carcinogenesis; conversely they may involve distinct mechanisms in the various *in vivo* paradigms tested so far. Manipulation of LTβR-signalling has revealed numerous aspects of their function in both health and disease, particularly in autoimmune diseases. There are various approaches to manipulate LTβR-signalling; one is stimulation with an agonistic receptor-specific monoclonal antibody (ACH6) or blocking with a soluble chimeric protein that efficiently inhibits LTβR-signalling (LTβR-Ig) (Force, W.R JI. 1995). LTβR-Ig has been so far the primary pharmacological tool to inhibit LTβR-signalling and HVEM. The construct binds to both LTαβ and LIGHT with a high affinity, consequently blocking the interactions of LTαβ with LTβR and LIGHT with both LTβR and HVEM, as outlined in **Figure 2** (Browning, 2008). Analysis of the effects of genetic or pharmacological disruption of the LTβR pathway in rodent models of inflammatory diseases and more recently in carcinogenesis has been extensively performed in the last years. **Table 2** lists the animal models where LTβR-Ig administration was efficacious.

Recent reports convincingly show an involvement of LTβR-signalling in inflammatory processes and carcinogenesis. For example LTβR-Ig treatment in TRAMP mice interrupted clonal T-cell deletion, reduced the size of the primary prostate cancer (CaP) and prevented further metastasis (Zhou et al., 2009) - suggesting a role of LT in controlling anti-tumor T-cell selection and primary cancer development. Moreover, depletion of LT expression on B-cells - not on T-cells - delayed CR-CaP (castration-resistant CaP) recurrence (Ammirante, 2010), pointing towards a therapeutic application of LTβR-signalling inhibition even as a tool to fight cancer re-emergence (Wolf et al., 2010). The chronic and relapsing course of many autoimmune diseases calls for new biological agents capable of suppressing the underlying inflammatory disorders. Recent studies indicate that inhibition of LTβR-signalling in adult

animals can prevent and relieve autoimmune diseases such as colitis, uveitis, Sjögren's syndrome, rheumatoid arthritis, insulin-dependent diabetes mellitus and experimental autoimmune encephalomyelitis EAE) (Gommerman and Summers deLuca, 2011).

Target	Disease	Model	References
LT signalling in cancer	Prostate cancer	TRAMP (SV40-Tag)	(Ammirante, 2010)
	Colon carcinoma	Syngeneic colon carcinoma tumor xenograft model	(Lukashev et al., 2006)
	Fibrosarcoma	LTβR-Ig expression on tumor cells	(Hehlgans et al., 2002)
	Hepatocellular carcinoma	AlbLTαβ mice	(Haybaeck et al., 2009)
	Nasopharyngeal carcinoma	Transplantation of tumors with amplification 12p13.3	(Or et al., 2009)
	Prostate cancer	TRAMP (SV40-Tag)	(Zhou et al., 2009)
LT signalling in infectious diseases	Prion disease	Scrapie infection	(Montrasio et al., 2000)
	Viral shock	LCMV infection	(Puglielli et al., 1999)
LT signalling in autoimmune diseases	Arthritis	Classical CIA	(Fava et al., 2003)
		Adjuvant arthritis	(Fava et al., 2003)
	Hepatitis	Con A hepatitis	(An et al., 2006; Anand et al., 2006)
		AlbLTαβ mice	(Haybaeck et al., 2009)
	Inflammatory Bowel Disease	Inflammatory $CD45RB^{hi}$	(Mackay et al., 1998)
		CD3εTg26	(Mackay et al., 1998)
		DSS	(Stopfer et al., 2004)
		TNBS	(An et al., 2005)
	Autoimmune pancreatitis	Tg(Ela1-LTa,b)	(Seleznik et al.)
	Multiple sclerosis	PLP_SJL EAE	(Columba-Cabezas et al., 2006; Gommerman et al., 2003)
		MBP-rat acute EAE	(Gommerman et al., 2003)
		Cuprizone	(Plant et al., 2007)
	Sjögren's syndrome	Male NOD	(Gatumu et al., 2009)
	Systemic Lupus Erythematosis	Adeno-IFN BWFI	Biogen Idec, unpublished
		Pristane SNFI	Biogen Idec, unpublished
		GVHD skin	(Wu et al., 2004)
		GVHD chronic	(Tamada et al., 2000; Tamada et al., 2002)
	Type I diabetes	Female NOD	(Ettinger et al., 2001 ; Lee et al., 2006 ; Wu et al., 2001)
	Uveitis	R16 immunization	(Shao et al., 2003)
LTα or LTαβ expressing T-cells	Arthritis	CIA	(Chiang et al., 2009)
	Delayed type hypersensitivity	KLH/CFA	(Chiang et al., 2009)
	Multiple sclerosis	EAE (MBP-TCR Tg mice)	(Chiang et al., 2009)

Table 2: Summary of experiments leading to disease regression in rodent models due to conditional interference with LTβR signalling (Adapted from Wolf et al. 2010)

There are three principal mechanisms for the potential mode of action in LTβR blockade. **Figure 4** summarizes the changes in the lymphoid microenvironment observed in mice following LTβR-Ig treatment (Spahn et al., 2005).

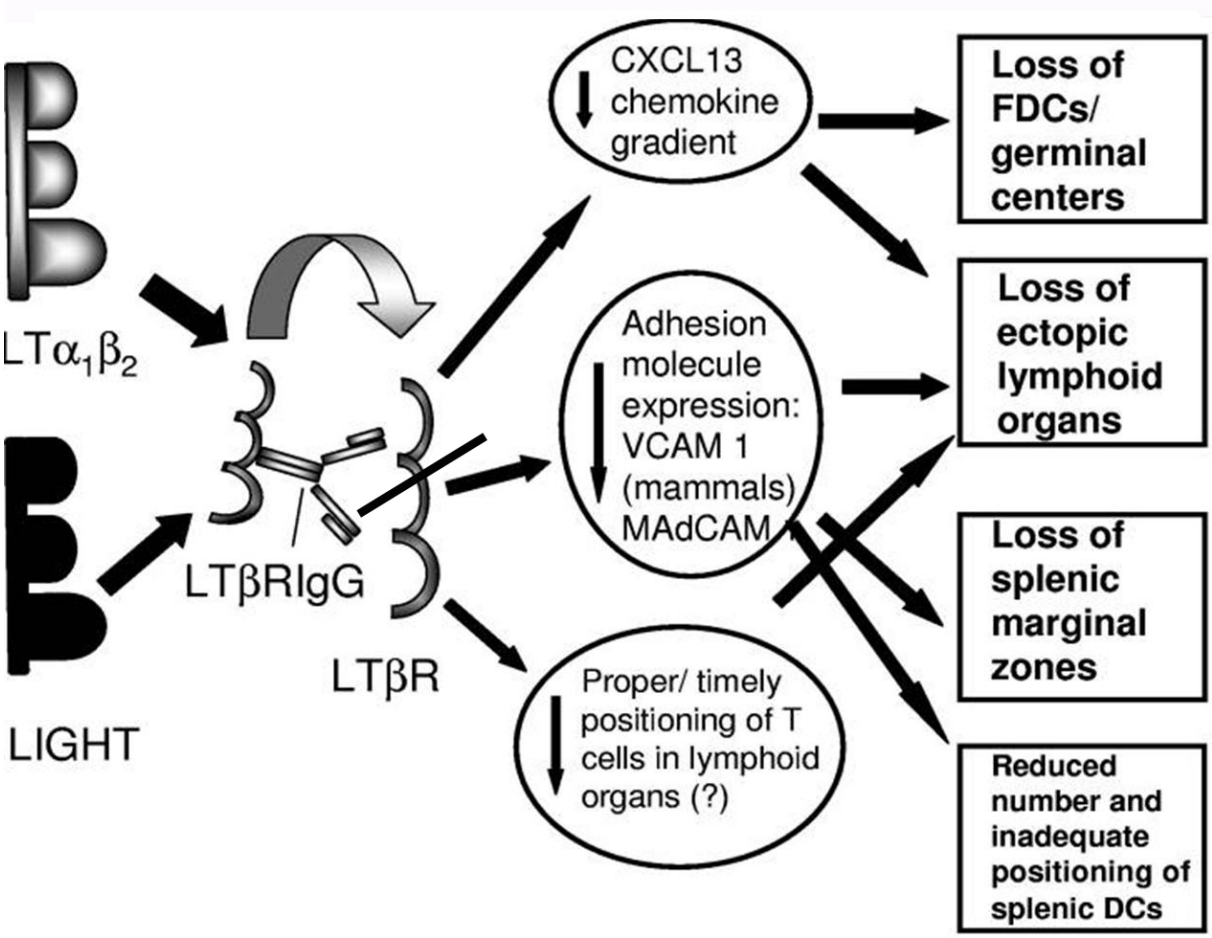

Figure 4. Proposed biological effects of LTβR pathway inhibition via LTβR-Ig. Three biological mechanisms (circles) have been described: loss of CXCL13 gradient in follicular dendritic cell networks, loss of adhesion molecule expression, and loss of proper and timely positioning of T cells in lymphoid organs. These mechanisms contribute to changes in secondary lymphoid organ formation, and loss of ectopic lymphoid organs, as indicated by the four boxes (Adapted from Spahn et al., 2005).

(1) Interruption of the positive feedback loop between LT and homeostatic chemokines, especially CXCL13. LTβR-signalling is required to maintain a CXCL13 chemokine gradient, which attracts $CXCR5^{+}$ B cells to the lymphoid follicle and to sustain the differentiation status of the recruited B-cells and the FDCs in the network. **(2)** Prevention of adhesion molecule (MadCAM-1, V-CAM1) expression, causing the absence of marginal zones. Similarly, LTβR engagement is required for continued expression of V-CAM1 by the FDC network (Gommerman and Browning, 2003) (Husson et al., 2000). Furthermore, by reducing

adressin expression, it can lead to diminished cell entry into LNs or TLOs (Browning, 2008) **(3)** Anti-LTβR treatment is able to impair immune function by preventing proper homing of T-cells, B-cells, and APCs in secondary lymphoid organs, thus preventing the induction of appropriate antigen-specific immune responses.

Hence, the observed pathological changes in mice treated with LTβR-Ig include the absence of discreet B-cell follicles, lack of FDC networks and the absence of marginal zones in the spleen. Furthermore, germinal centers did not form in these mice upon immunization.

As many autoimmune diseases are accompanied by the formation of pathological ectopic-lymphoid structures, TLOs, the inhibition of LTβR pathway has a potential to be used as an alternative or supplementary approach in treatment of autoimmune diseases.

2. A Lymphotoxin-driven, IL17-independent pathway to autoimmune pancreatitis

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2.1 Abstract

The mechanisms triggering autoimmune pancreatitis (AIP), an increasingly recognized, immune mediated form of chronic pancreatitis, remain elusive. Current treatment options are limited, frequently resulting in disease relapse. Here we report upregulation of lymphotoxin (LTαβ) in pancreata of AIP patients concomitant with elevated pancreatic and serum chemokine levels - a signature unaffected by corticosteroid treatment and contributing to relapse. Acinar-specific LT expression in mice (*Ela1-LTa)* induced autoimmunity reminiscent of human AIP, including ANA, auto-antibodies against pancreatic self-antigens and renal immune-complexes, causally linking pancreatic LTαβ expression with AIP. Notably, chronic inflammation initially and exclusively arising in the pancreas sufficed to induce a systemic autoimmune disease. Disease mechanisms of AIP development and potential therapies were tested in *Ela1-LTab* mice. Absence of lymphocytes *(Ela-1LTab/Rag1$^{-/-}$)* abrogated AIP, while lack of pro-inflammatory monocytes *(Ela-1LTab/Ccr2$^{-/-}$)* eliminated early pancreatic tissue damage but failed to prevent AIP. Adoptive transfer of transgenic splenocytes into *Rag1$^{-/-}$* mice suggested that acinar cell damage in *Ela1-LTab* mice is T-cell mediated. Corticosteroid treatment reduced pancreatitis but did not hamper auto-antibody production. In contrast, inhibition of LTβR-signalling dampened chemokine expression, renal immune-complex deposition and fully abrogated AIP. Thus, suppression of LTβR-signalling might become a viable alternative or combinatorial treatment option for AIP.

2.2 Introduction

Chronic pancreatitis (CP) is an inflammatory disease of the exocrine pancreas leading to irreversible and progressive tissue damage resulting in severe exocrine and endocrine insufficiency (Braganza et al., 2011). Chronic destruction of acinar cells usually coincides with inflammation, metaplasia and fibrosis. Various aetiologies induce CP e.g. chronic alcohol intake, smoking and drug abuse (Talamini et al., 1996; Yadav and Whitcomb, 2010). Notably, chronic pancreatitis is believed to increase the risk to development of pancreatic ductal adenocarcinoma (PDAC) (Guerra et al., 2011). Autoimmune pancreatitis (AIP) is a distinct form of CP with so far unknown aetiology, but with characteristic clinical and histological features (Kloppel et al., 2003). It is speculated that 11-15% of all CP cases develop in an autoimmune context (Finkelberg et al., 2006). Two distinct types of AIP have been described: (1) Type 1 AIP, with lobular and interlobular inflammation, germinal center

formation, prominent lymphoplasmacytic inflammation and vasculitis, in which the pancreas is involved in a systemic IgG4-positive disease. (2) Fibroinflammatory duct-centric type (type 2 AIP) with granulocyte epithelial pancreatic lesions and destruction of the pancreatic duct without IgG4-positive cells or systemic involvement (Park et al., 2009).

In the clinic, elevated IgG4 levels are currently the most sensitive serum parameter used for diagnosis (Hamano et al., 2001). However, about half of all AIP patients have normal serum IgG4 levels, and elevated IgG4 levels were also reported in non-autoimmune pancreatitis and pancreatic cancer (Choi et al., 2007). Recently, B-cell activating factor (BAFF) was identified as a novel serum marker for diagnosis and treatment response of AIP in combination with total serum IgG and IgG4 or anti-nuclear antibodies (ANA) (Yamanishi et al., 2011). However, the identification of further selective diagnostic markers remains a challenge.

Auto-antibodies detected in human AIP, are anti-carbonic anhydrase II (anti-CA-II), anti-lactoferrin (anti-LF), and more recently, anti-trypsinogen antibodies (Lohr et al., 2010). Current studies revealed that approximately 40% of AIP patients suffer from extra-pancreatic manifestations involving liver, salivary glands, retro-peritoneum or kidneys (Kamisawa et al., 2006), (Raina et al., 2009). Thus, AIP is a systemic autoimmune disease, which responds well to conventional steroid treatment (Finkelberg et al., 2006); however relapses are frequent (~ 40%) and are associated with a more severe disease course (Kalaitzakis and Webster, 2011). Hence, novel therapeutic strategies and a better understanding of the underlying disease mechanisms are needed.

Major regulators of immunity under both normal and pathological conditions include the cytokines LTα, LTβ and their receptor (LTβR), members of the tumor necrosis factor (TNF) superfamily. Under physiological conditions, LT is expressed mainly by immune cells. Under pathological conditions, parenchymal cells - like hepatocytes - are also capable of expressing LT in mice and humans (Haybaeck et al., 2009), (Lowes et al., 2003). LTβR-signalling serves pleiotropic functions including control and maintenance of lymphoid organ development (Gommerman and Browning, 2003), development of high endothelial venules (HEVs) (Browning et al., 2005) and control of lipid homeostasis (Lo et al., 2007). LT is crucial for the generation and maintenance of tertiary lymphoid organs (TLOs), frequently arising during chronic inflammation (Haybaeck et al., 2009), (Drayton et al., 2003), (Kratz et al., 1996). TLOs are found in many human autoimmune diseases (e.g. rheumatoid arthritis, Sjögren's syndrome, Multiple sclerosis) (Takemura et al., 2001),(Hjelmervik et al., 2005),(Serafini et al., 2004). TLOs upregulate LT, express chemokines and adhesion molecules (e.g. CXCL13, CCL21; V-CAM) and contain cells or structures known to depend on LTβR-signalling (e.g.

follicular dendritic cells (FDC), HEVs) (Browning et al., 2005),(Browning, 2008; Drayton et al., 2006; Drayton et al., 2003; Heller et al., 2007). The consequences of ectopic LT expression were previously investigated in several transgenic animal models, such as RIP-LTα (Picarella et al., 1992), AlbLTαβ (Haybaeck et al., 2009) and lckLTαβ (Heikenwalder et al., 2008). Although these models display chronic inflammation and TLOs in the respective organs, they did not develop detectable organ-specific or systemic autoimmune disease.

Characterization of human AIP tissue revealed strong up-regulation of the cytokines LTαβ and its target genes but not IL17 as recently proposed to be important in TLO induction in non-lymphoid organs (Rangel-Moreno et al., 2011). Based on this data and on the known role of LTβR-signalling in immunity and disease, we aimed to test the potential role of LT in AIP pathology. To this end, we created transgenic mice expressing LT under the control of the acinar-cell specific elastase promoter (*Tg*(Ela1-LTa,b). Strikingly, *Tg*(ELa1-Lta,b) mice reproduced a number of key pathological hallmarks of human AIP, demonstrating for the first time that chronic inflammation which initially develops exclusively in the pancreas suffices to induce AIP and a systemic autoimmune disease. Moreover, *Tg*(ELa1-Lta,b) mice serve as a novel mouse model to study disease development, pathogenesis and potential treatment interventions for AIP. Here, we have utilized *Tg*(Ela1-LTa,b) mice to investigate AIP disease mechanisms, determine the source of AIP relapse after corticosteroid treatment, and to explore novel therapeutic strategies for AIP.

2.3 Results

2.3.1. Distinct expression patterns of TNFR-superfamily members and chemokines in non-AIP versus AIP patients

We first investigated whether particular subsets of cytokines or chemokines are deregulated in pancreata of patients suffering from pancreatitis. We analyzed frozen material derived from inflammation-unaffected patients (n=6), patients with pancreatic inflammation with different degrees of fibrosis (n=6), as well as patients with AIP (n=5) by qPCR analysis. Identical tissue specimens used for mRNA expression were pre-screened histologically by Hematoxilin-Eosin (H&E) staining to verify the respective pancreatic pathology (**Figure 5**).

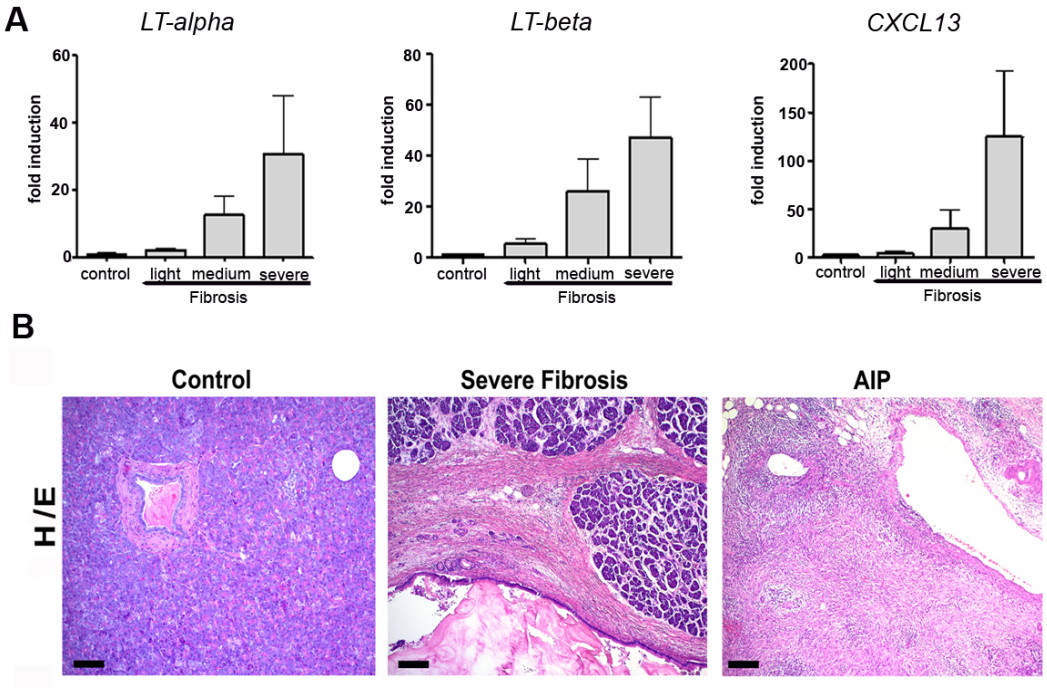


Figure 5. **Characterization of human autoimmune pancreatitis and non-autoimmune pancreatic inflammatory diseases. (A)** *LT–alpha LT-beta* and *Cxcl13* transcripts were analysed in pancreatic tissue samples of patients with non-AIP of different fibrosis degree (light (stage I), medium (stage II) and severe fibrosis (stage III)) and compared to unaffected pancreatic tissues. The staging of fibrosis was carried out based on the amount of syrius red positive areas in the tissue sample. **(B)** Histological analysis of representative human pancreatic tissues from a healthy control patient (scale bar: 100 μm), a patient with pancreatic inflammatory disease with severe fibrosis (scale bar: 200 μm) and an autoimmune pancreatitis (AIP) case (scale bar: 100 μm). H/E staining shows typical features of pancreatic inflammation (fibrosis) and autoimmune disease (inflammatory infiltrates, vasculitis).

mRNA expression analysis revealed strong and significant upregulation of *LTα, LTβ, CXCL13*, CCL19, *CCL21*, *CCL17* and *BAFF* in AIP patients when compared to controls and patients with severely fibrotic non-autoimmune pancreatic inflammation. A possible candidate cytokine that was recently proposed to be crucial for autoimmune disease development, (e.g. rheumatoid arthritis, psoriasis, multiple sclerosis, and inflammatory bowel diseases (Yamada, 2010)) as well as for TLO generation in non-lymphoid organs of mice (e.g. iBALT) is IL17 (Rangel-Moreno et al., 2011). However, we could not detect a significant induction of IL17 in pancreata of human AIP patients. In contrast, increase in *CCL20*, *TNFα, CCL2*, *IL6*, *IL17* and *α-SMA* transcripts were found in fibrotic, non-AIP pancreata when compared to healthy controls or AIP patients **(Figure 6)**. This reveals distinct transcriptional signatures in pancreata of AIP and non-AIP patients with remarkably high expression of LT and homeostatic chemokines (e.g. CXCL13, CCL19, CCL21), especially in AIP.

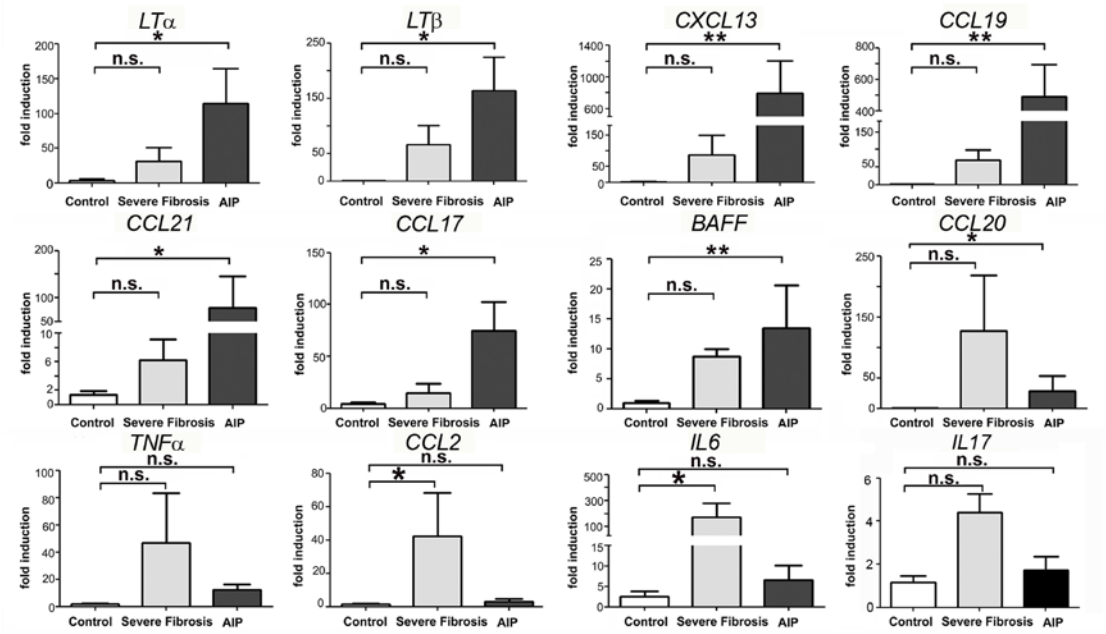


Figure 6. **Distinct transcriptional signature in AIP and CP patients.** qPCR analysis of human cryo-material from pancreatic tissues for *LTa* and *LTb* transcripts, homeostatic chemokines and LT target genes (e.g. *CXCL13, CCL19, CCL21, CCL17, CCL20*) as well as inflammatory mediators (*BAFF, TNFα, CCL2, IL6,, IL17*).

2.3.2. LT expression is found on infiltrating lymphocytes and metaplastic acinar cells

We next aimed to identify the cells expressing LT in the pancreata of CP and AIP patients. Immunohistochemistry of pancreatic tissue sections derived from non-AIP patients with fibrotic pancreatitis (n=12), from AIP patients (n=16) compared to unaffected control tissue

(n=6) revealed strong LTβ protein expression on infiltrating lymphocytes and acinar cells (**Figure 7**). In most cases, the latter were undergoing acinar-to-duct-metaplasia. Double-staining with lymphocytic markers CD3 (T-cells), CD20 (B-cells) as well as amylase (acinar cells) supported this finding. Furthermore, the frequency of $LT\beta^{+}CD20^{+}$ B-lymphocytes appeared to be strongly increased in AIP patients compared to patients suffering from fibrotic pancreatitis, which primarily display LTβ expression on T-cells.

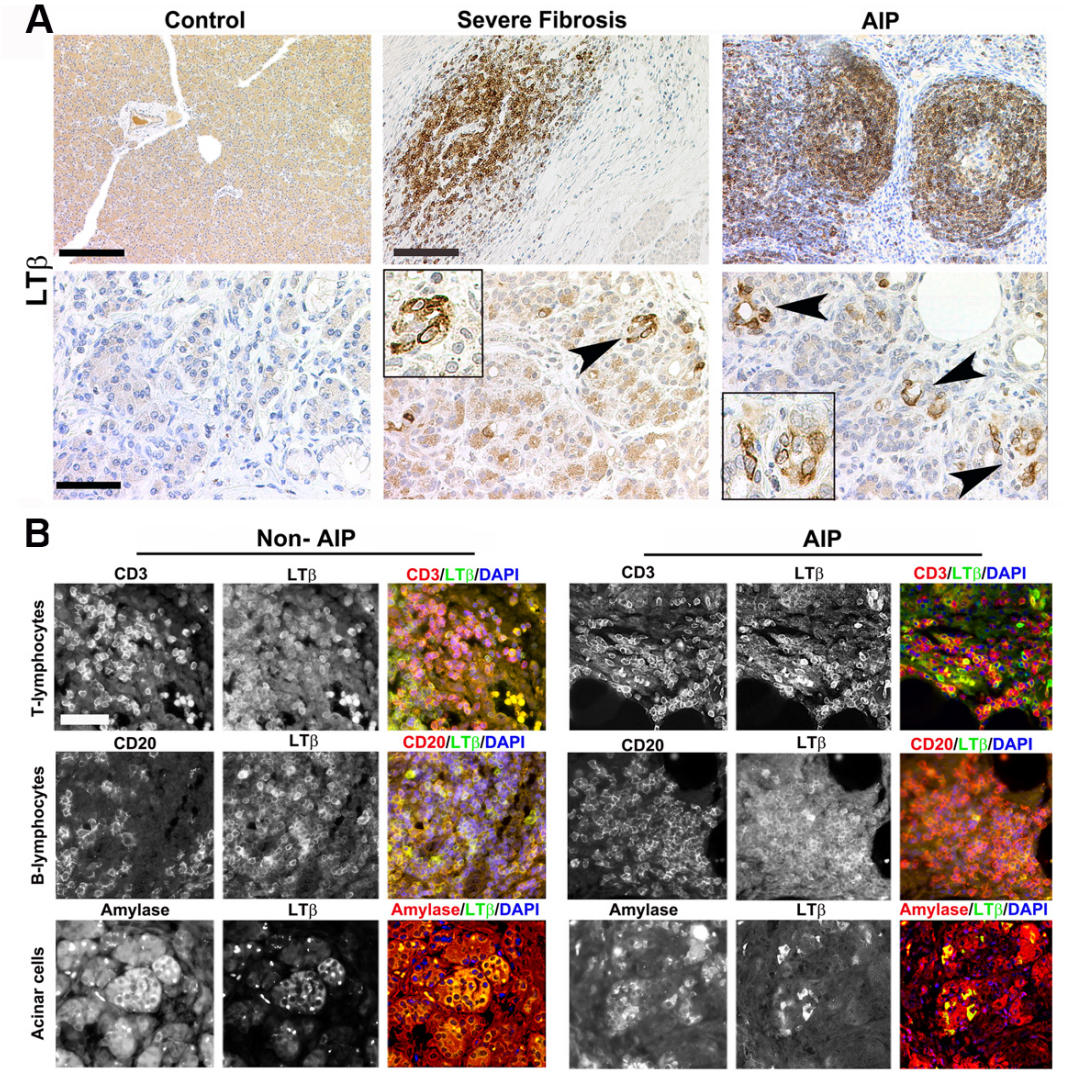


Figure 7. **Pancreatic lymphotoxin expression of healthy individuals, CP and AIP patients . (A)** Human LTβ expression on inflammatory cells as well as on acinar cells - especially that undergoing acinar-to-duct metaplasia (insets). Size of scale bars: Upper row: Control: 200μm. Severe fibrosis and AIP: 100 μm. Lower row: Control, severe fibrosis and AIP: 50 μm. **(B)** LTβ is expressed on $CD3^{+}$ T-cells, $CD20^{+}$ B-cells and on $amylase^{+}$ acinar cells in pancreatitis from both autoimmune and non-autoimmune origin. Of note, whereas more T cells and a sub-fraction of B cells express LTβ in pancreatic inflammatory disease with severe fibrosis (non-AIP), AIP patients express LTβ to high degree on almost all B cells and less on T cells (scale bar: 50 μm).

2.3.3. Serum analysis of healthy individuals and AIP patients

The observed induction of various cytokine and chemokine transcripts was also detectable in serum of AIP patients (**Figure 8**). We found homeostatic chemokines (CXCL13, CCL19, CCL21) and pro-inflammatory cytokines (TNFα, IL6) strongly elevated in sera of AIP patients when compared to healthy individuals. We next investigated whether these chemokines are modulated upon corticosteroid treatment in AIP patients (n=10). Application of standard corticosteroid treatment for 12 weeks did not lead to significant reduction of the investigated cytokines and chemokines. Therefore, although corticosteroid-treatment significantly reduced some indicators of inflammation (IgG, IgG4), levels of important inflammatory mediators remained insignificantly changed, indicating a possible cause of disease re-emergence.

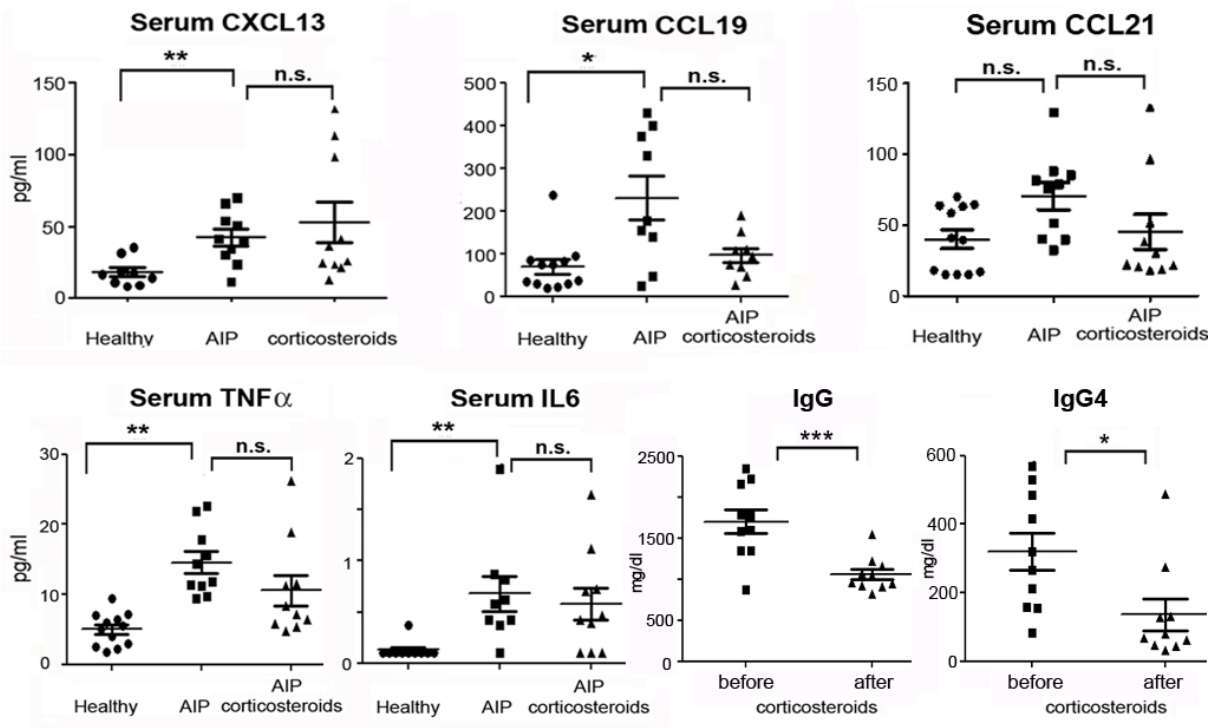


Figure 8. **Serum analysis of healthy individuals and AIP patients.** Serum from healthy controls and AIP patients - before and after corticosteroid treatment – was analysed for the presence of CXCL13, CCL19, CCL21, TNFα and IL6 proteins. Serum IgG and IgG4 is significantly decreased in AIP patients treated with corticosteroids for 12 weeks.

2.3.4. Generation and molecular characterization of *Tg*(ELa1-Lta,b) mice

Data presented above indicate a distinct cytokine/chemokine signature in fibrotic pancreatitis and AIP patients. Moreover, focal LT expression on metaplastic acinar cells and the correlation between LT upregulation suggested a link between LT expression and AIP. Hence, we aimed to test whether ectopic LTαβ expression in the murine exocrine pancreas would induce tissue damage and AIP in mice.

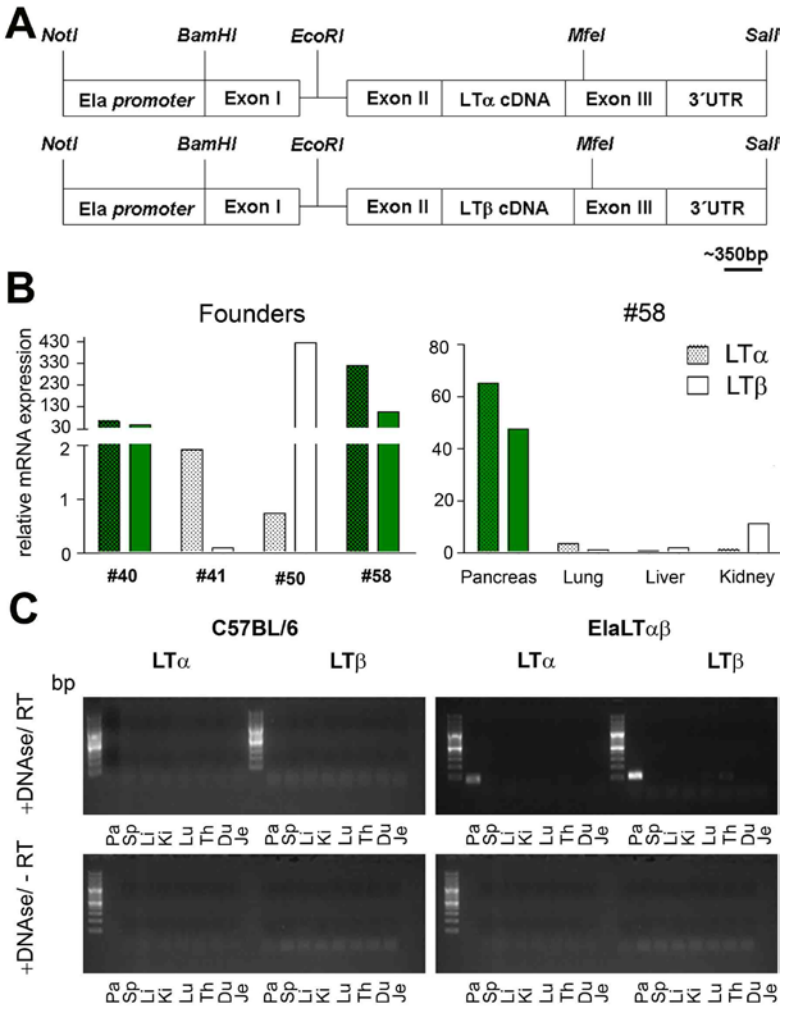


Figure 9. Generation of transgenic mice. (**A**) Schematic representation of the transgenic LTα and LTβ constructs controlled by the rat elastase 1 promoter, co-injected into C57BL/6N zygotes. (**B**) TaqMAN-PCR analysis revealed *Tg(ELa1-Lta,b)* line #58 as the higher and *Tg(ELa1-Lta,b)* line #40 as the lower expresser double transgenic line. LTαβ expression is highly specific to the pancreas. No or very little (>100 fold) elevated LTαβ mRNA expression was found in lung, liver or kidney compared to C57BL/6 tissues. (**C**) Analysis of transgene specific LTαβ mRNA expression in pancreas, spleen, liver, kidney, lung, thymus, duodenum and jejunum of transgenic mice and negative littermates. PCR was performed on cDNA isolated from 8 week-old mice using primers specific for transgenic *Ela1-Lta Ela-Ltb* constructs. Similar to TaqMan data, no transgenic mRNA expression was detected by this method in other organs than pancreas.

We therefore generated double transgenic mice expressing LTα and LTβ in the pancreas under the control of the rat elastase 1 promoter (Swift et al., 1984). Upon PCR analysis, we selected two double transgenic, LTab expressing lines, denoted as line #40 and #58, for further experiments. Southern blot analysis confirmed the integration of two copies of LTα and β for line #58 or one copy of each construct per haploid genome for line #40. After thorough Real-time PCR analysis of various organs derived from lines #40 and 58, we verified that transgene expression is restricted to the pancreas, and no significant LT expression could be detected in the spleen, thymus, lung, liver, kidney, jejunum or duodenum (**Figure 9**).

Transgene expression on mRNA and LTα protein level differed between the two transgenic lines, thus line #40 was classified as the low expressing and line #58 as the high expressing (**Figure 9B and Figure 10**). *Tg(ELa1-Lta,b)* henceforth refers to the high expressing line #58, however, we have corroborated the observed phenotypes also in mice of line #40.

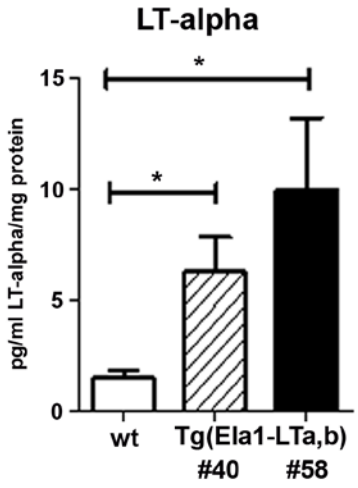


Figure 10. Protein expression level of LTα in pancreata. Lymphotoxin alpha protein levels of *Tg(ELa1-Lta,b) line* #58 and #40 measured by ELISA confirmed transcription analysis.

2.3.5. Chronic pancreatitis and altered amylase, lipase levels in *Tg*(ELa1-Lta,b) mice

We first analysed whether ectopic LT expression in acinar cells would lead to tissue damage indicated by increased pancreatic enzyme levels (e.g. amylase; lipase) in sera of transgenic mice. *Tg(ELa1-Lta,b)* mice showed strongly and significantly elevated levels of both amylase and lipase already by the age of one month (**Figure 11A**).

To investigate macroscopic changes, pancreas to body weight ratio was calculated in three, six and twelve month-old mice (**Figure 11A**). Pancreas size was significantly smaller (30-40%) in *Tg(ELa1-Lta,b)* mice compared to wild type mice at all time-points. This finding

could be explained by the prominent accumulation of inflammatory cells persisting at later time points.

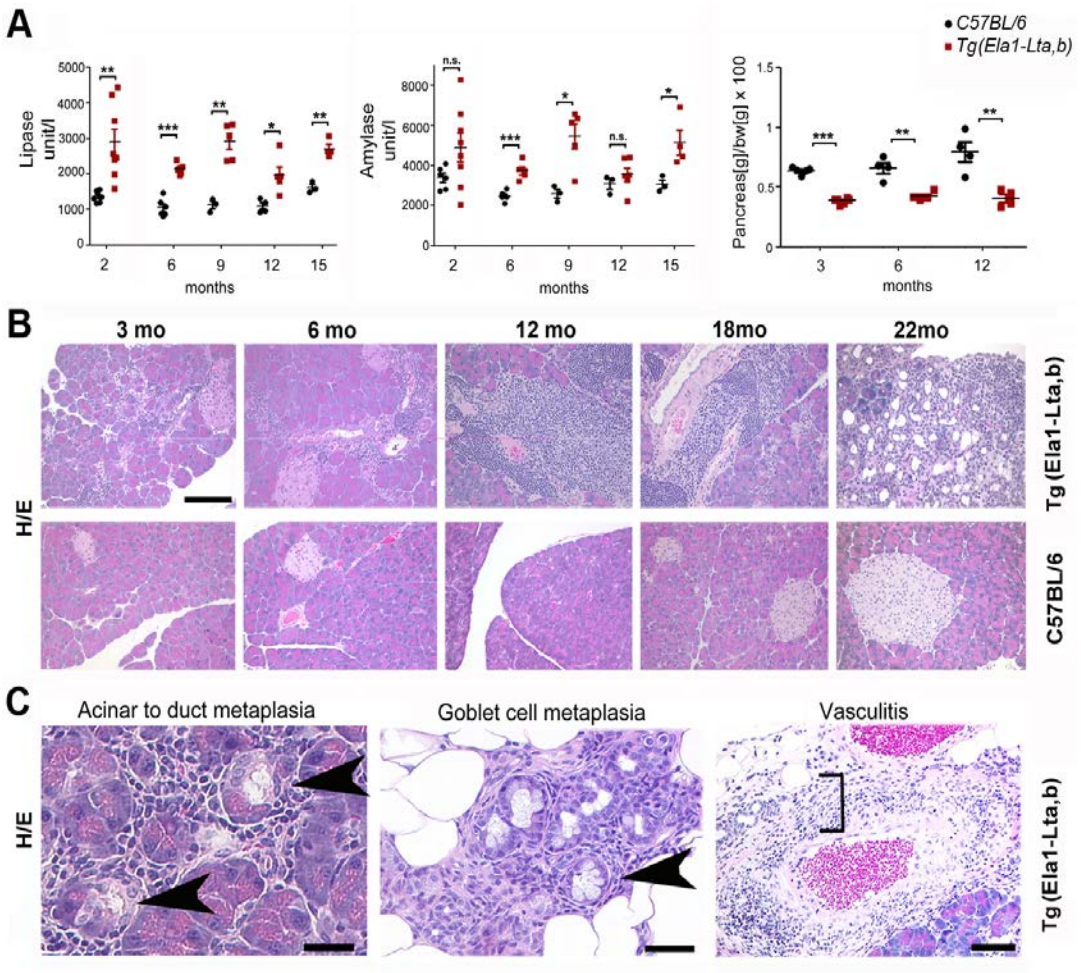


Figure 11. Description of inflammatory phenotype in *Tg*(Ela1-Lta,b) mice. (A) Pancreatic enzyme levels of lipase and amylase in serum were elevated starting from the age of two months in *Tg(ELa1-Lta,b)* mice. Changes in size and weight of the pancreas as visualized by pancreas to body weight ratio shows significant decrease in transgenic mice compared to wild type mice. **(B)** Development and progression of pancreatic inflammation as visualized by H/E staining from 3, 6, 12, 18 and 22 months old *Tg(ELa1-Lta,b) line* #58 mice compared to negative littermates (scale bar: 100 μm). **(C)** Typical features of chronic pancreatitis like acinar to duct metaplasia (scale bar: 20 μm), goblet cell metaplasia (Scale bar: 50 μm) and vasculitis (scale bar: 100 μm) were found in *Tg(ELa1-Lta,b)* mice.

Based on the early acinar cell damage reflected by elevated pancreatic serum enzyme levels, we addressed whether ectopic LT expression on acinar cells induces inflammation and tissue damage (**Figure 11B**). In both transgenic lines, first focal infiltrating immune cells (T, B lymphocytes and F4/80^{+}) accumulated in the pancreas already by eight weeks of age accompanied by an early onset acinar-to-duct-metaplasia.

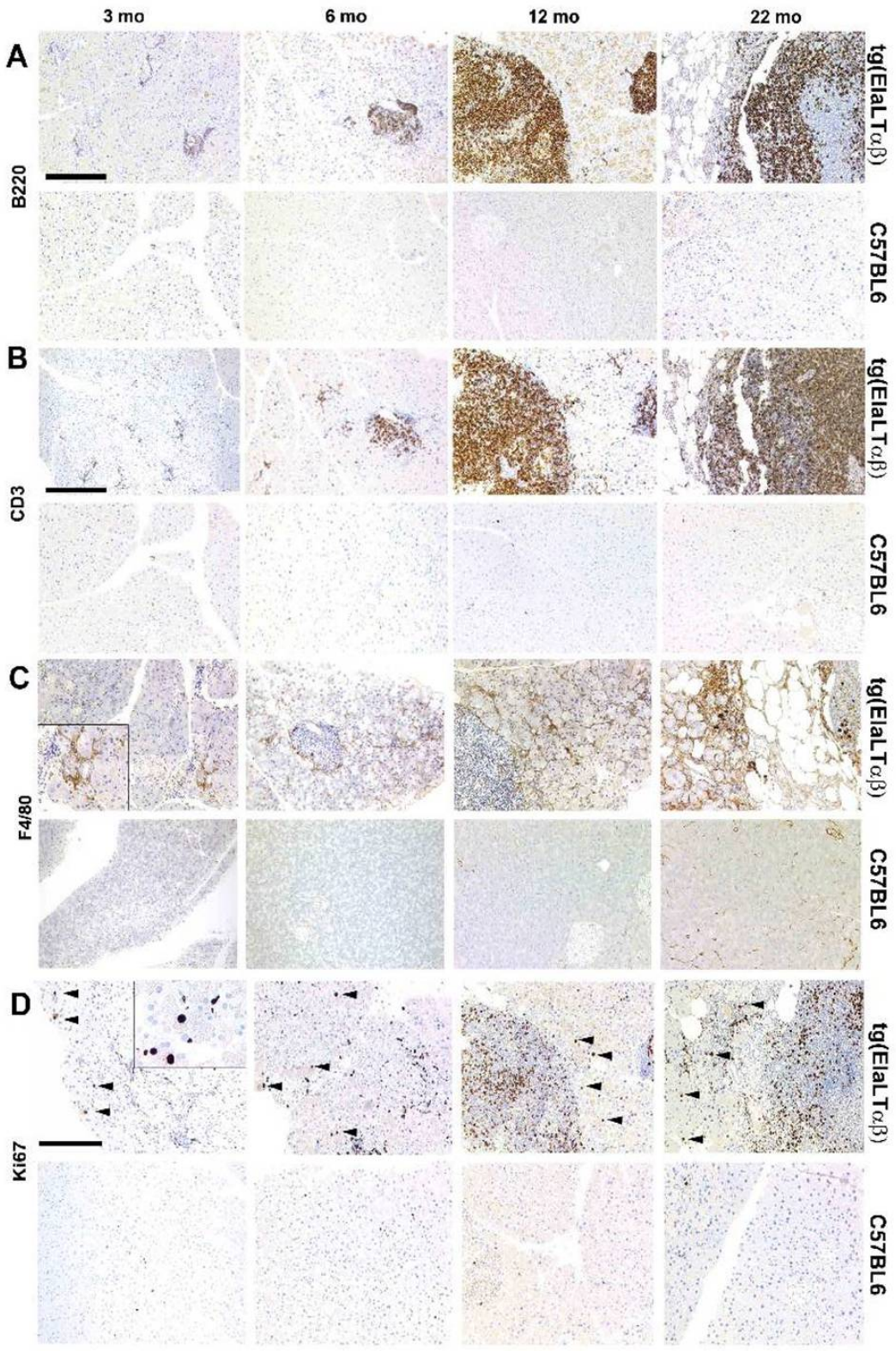


Figure 12. Immunohistochemical analysis of lymphocytes, macrophages and proliferative cells in *Tg(ELa1-Lta,b)* pancreata over time. (a) $B220^+$ B-cells, **(b)** $CD3^+$ T-cells, **(c)** $F4/80^+$ macrophages (see also inset) **(d)** and $Ki67^+$ proliferating inflammatory cells and acinar cells (see also inset, arrow heads) were stained in *Tg(ELa1-Lta,b)* mice compared to negative littermate controls. $Ki67^+$ proliferating inflammatory and acinar cells are indicated (arrow heads). Scale bar: 200 μm.

Pancreatitis progressed over time, with a strong influx of inflammatory cells and concomitant proliferation of Ki-67^{+} acinar cells to pronounced tissue damage at 12-22 months of age (**Figure 12**).

The constantly rising number of immune cells led to development of organized B- and T-cell zones after 6 months, indicative of the formation of tertiary lymphoid organs. Sporadic acinar-to-goblet cell metaplasia and vasculitis were also observed - two histo-pathological features commonly found in human CP and AIP (**Figure 11C**). Inflammation was restricted to the exocrine part of the pancreas; in contrast pancreatic islets remained unaffected (**Figure 11B and Figure 13 B**). The body weight of transgenic mice did not differ significantly from wt littermates at any time point (**Figure 13A**). Based on the comparable patterns of pancreatic inflammation observed in both independent transgenic lines (**Figure 13B**), the phenotype of *Tg(ELa1-Lta,b)* mice is unlikely to be due to a positional effect of randomly integrated transgenes.

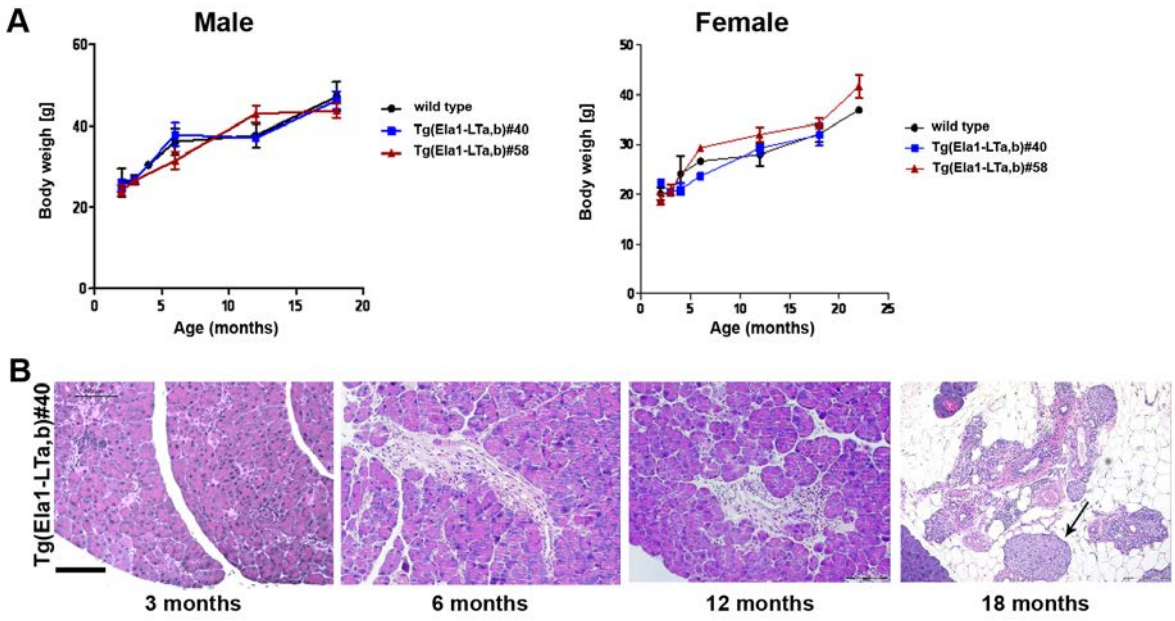


Figure 13. Characterization of both transgenic lines. (A) Body weight was measured from both males and females in *Tg(ELa1-Lta,b) line* #58 and *Tg(ELa1-Lta,b) line* #40 at 2, 3, 6, 12 and 18 months of age. No significant difference was found either for male or female transgenic mice when compared to age matched wild type controls. **(B)** Development and progression of pancreatic inflammation in the *Tg(ELa1-Lta,b) line* #40 visualized with H/E staining at the age of 3 months, 6 months, 12 months and 18 months. Lower LT expression leads to less infiltrating inflammatory cells, but the formation of tubular complexes after 18 months is comparable to the higher expresser transgenic line. Scale bar: 100μm.

2.3.6. *Tg*(ELa1-Lta,b) mice display a transcriptional signature reminiscent of human AIP

Next, we investigated mRNA and protein expression of chemokines and cytokines in pancreata of three- and twelve-month-old transgenic mice compared to negative littermates (**Figure 14A**). Not only a rise in endogenous *Lta* and *Ltb* transcripts over time but also an induction of the homeostatic chemokines *Cxcl13*, *Ccl19*, *Ccl21*, *Ccl17*, *Ccl20*, the adhesion molecule *Vcam*, the inflammatory chemokines *Cxcl1*, *Cxcl10*, *Ccl2*, the inflammatory cytokines *Baff*, *Il6*, *Il1β*, *Il7* and *Tnfα* was detected.

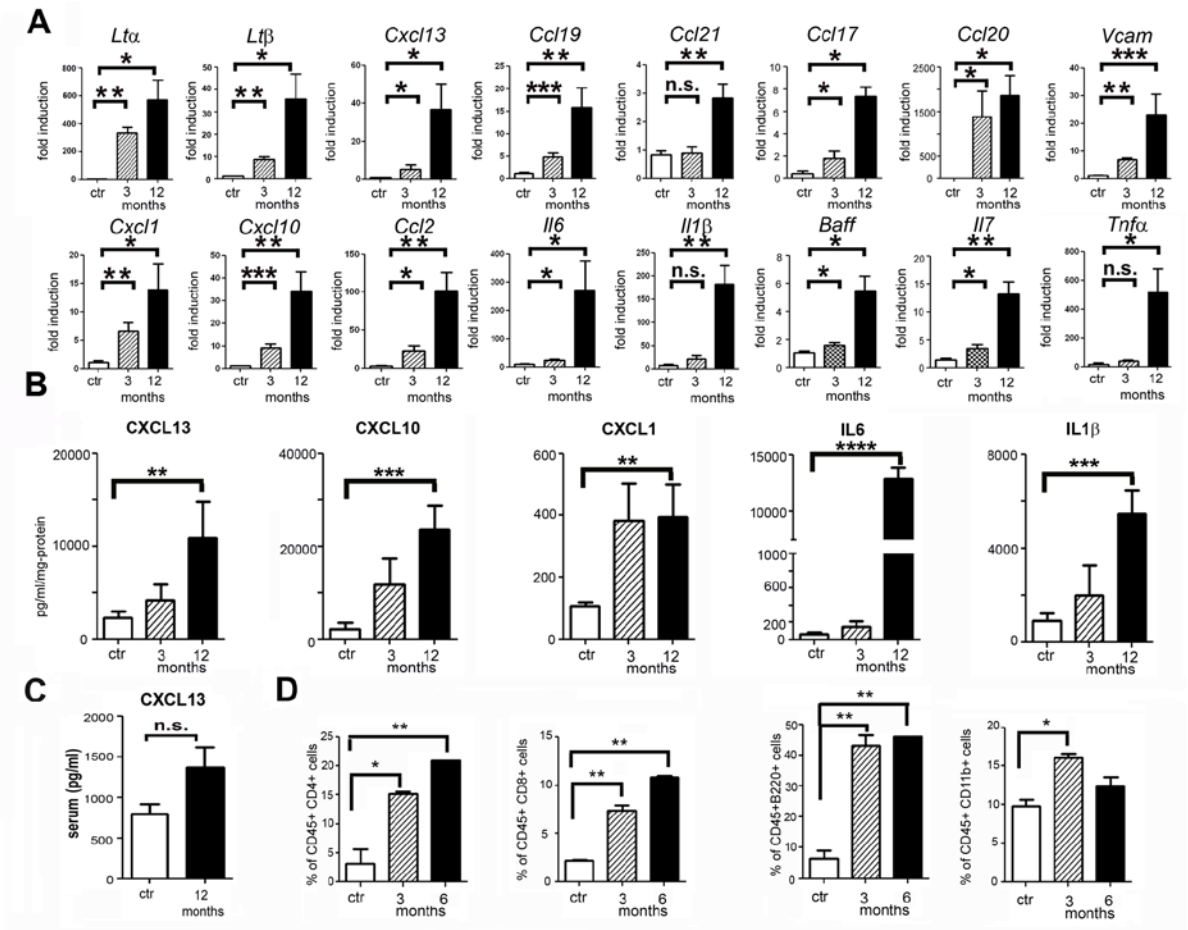


Figure 14. Cellular and molecular characterization of *Tg(ELa1-Lta,b)* mice. (A) Quantitative RT-PCR analysis of pancreatic *Lta*, *Ltb*, *Cxcl13*, *Ccl19*, *Ccl21*, *Ccl17*, *Ccl20*, *Vcam*, *Cxcl1*, *Cxcl10*, *Ccl2*, *Il6*, *Il1β*, *Baff (Tnfsf13b)*, *Il7* and *Tnfα*, transcripts in 3 and 12 month-old *Tg(ELa1-Lta,b)* pancreata compared to negative littermates (3 and 12 months pooled). **(B)** Elisa for CXCL13, CXCL10, CXCL1, IL6 and IL1β in pancreas homogenates of C57BL/6 and *Tg(ELa1-Lta,b)* mice. **(C)** Serum CXCL13 levels are elevated in 12 month-old *Tg(ELa1-Lta,b)* (n=10) mice compared to age matched C57BL/6 (n=10) mice. **(D)** Quantification of $CD4^+$, $CD8^+$, $B220^+$ and $CD11b^+$ infiltrating cells at the age of 3 and 6 months in transgenic and wild type mice.

This resembles the development of a progressive inflammatory response concomitant with the formation of a homeostatic environment favourable to the development of TLOs. Transcriptional data were corroborated by pancreatic protein expression of CXCL13, CXCL10, CXCL1, IL6 and IL1β (**Figure 14B**). Similar to human AIP patients, serum protein levels for CXCL13 were elevated in transgenic mice (**Figure 14C**).

To gain a quantitative and qualitative overview of the immune cell populations in transgenic mice at the age of three and six months, we performed flow cytometry on isolated white blood cells from pancreata of C57BL/6 and *Tg(ELa1-Lta,b)*. Consistent with the histological evaluation, a drastic increase in $CD4^+$, $CD8^+$ and $B220^+$ lymphocytic cells in transgenic pancreata was shown at both time-points (**Figure 14D**). In contrast, control littermates displayed almost no detectable $CD4^+$, $CD8^+$ and $B220^+$ cells.

Moreover, we found a strong increase in $CD45^{hi}CD11b^{mi}$ (R1) and $CD45^{hi}CD11b^{hi}$ (R2) cells in pancreata of *Tg(ELa1-Lta,b)* mice compared to negative littermates. Further analyses revealed that the $CD45^{hi}CD11b^{mi}$ population contained primarily granulocytes ($Ly6C^{hi}Ly6G^{hi}$ cells) (approx. 73%) and to a lower degree inflammatory monocytes ($Ly6C^{hi}$ $Ly6G^{lo}$) (approx. 27%). The $CD45^{high}CD11b^{high}$ cells were mainly composed of Ly-$6C^{low}$ and Ly-$6G^-$ cells, which are most likely resident macrophages (**Figure 15**).

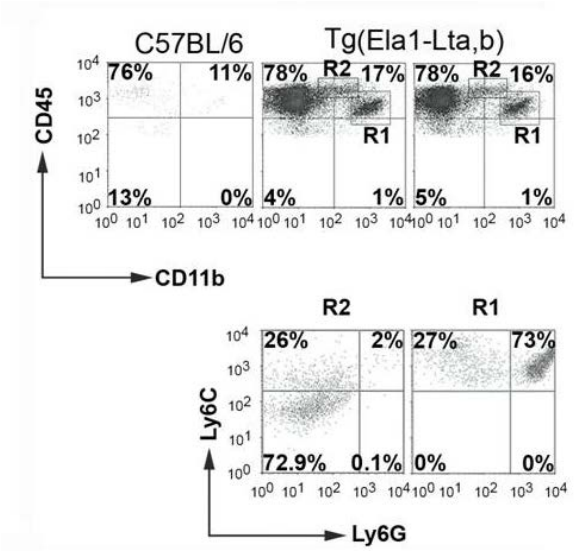


Figure 15. Characterization of myeloid cells in *Tg(ELa1-Lta,b)* mice. Two $CD11b^+$ populations were found in pancreata of 3 months old transgenic mice: *R1* depicts $CD11b^{high}$ and R2 $CD11b^{low}$ cells. Gated on the R1 population in average 73% are $Ly6C^+$ $Ly6G^+$ granulocytes and 27% $Ly6C^+$ inflammatory monocytes. In the R2 population $Ly6C^{low}$ $Ly6G^{neg}$ cells were identified as resident macrophages.

Although β-pancreatic islets were not directly affected by the inflammatory process, pancreatic inflammation can lead to endocrine insufficiency. Therefore, we tested whether Tg mice are diabetic. Even though *Tg(ELa1-Lta,b)* mice remained tolerant in response to glucose load during an intraperitoneal glucose tolerance test (IPGTT,; (Khasawneh et al., 2009) **Figure 16A**), they had to secrete ~ 2-3 fold more insulin than their control littermates in order

to support the same glucose clearance. Further, qPCR analysis of genes involved in glucose and lipid metabolism revealed deregulation of transcripts involved in lipolysis, fatty acid oxidation, gluconeogenesis and insulin signalling were indicative of alterations in the endocrine compartment (**Figure 16B**). Overall, glucagon- and insulin-producing α and β cells in pancreatic islets appeared normal arguing against a functional deficit that could give rise to diabetes in *Tg(ELa1-Lta,b)* mice (**Figure 16C**).

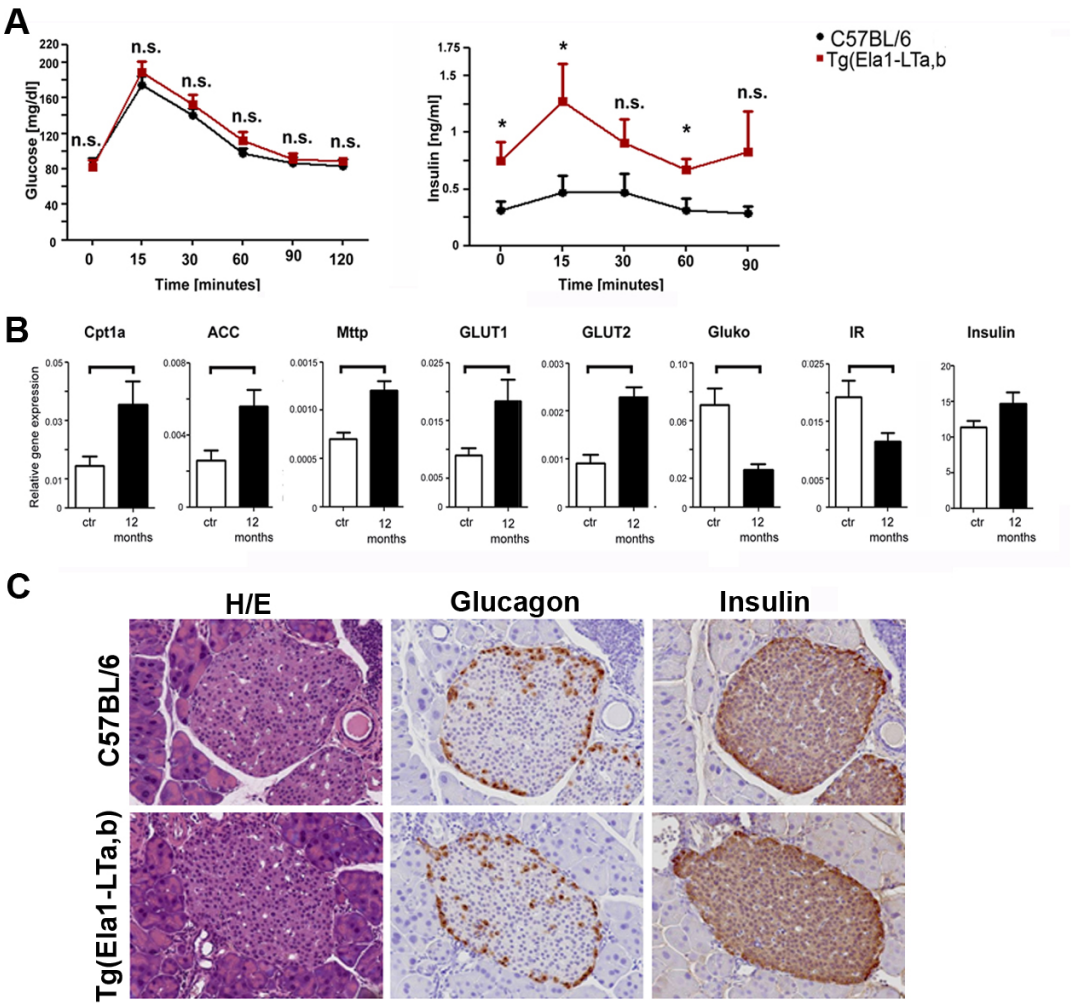


Figure 16. Analysis of the endocrine function of the pancreas in *Tg(ELa1-Lta,b)* and control mice (A) Intraperitoneal glucose tolerance test (IP-GTT) of 12 month-old *Tg(ELa1-Lta,b)* and C57BL/6 mice shows no signs of diabetes in *Tg(ELa1-Lta,b)* mice, however plasma insulin levels are significantly higher throughout IP-GTT. **(B)** Analysis of pancreatic *Cpt1a*, *Acc*, Mttp, *Glut1*, *Glut2*, *Gluko*, *insulin receptor* (*Ir*) and *insulin* transcripts by Real-time PCR of 12 month-old *Tg(ELa1-Lta,b)* mice compared to negative littermates. **(C)** Immunohistochemical analysis of pancreatic islets in 12 months old *Tg(ELa1-Lta,b)* mice and in age matched C57BL/6 mice. No difference in insulin or glucagon expression can be seen on pancreatic islets of transgenic mice compared to control. Magnification: 20x.

2.3.7. Germinal center formation and increased regulatory T-cells in *Tg(ELa1-Lta,b)* mice

To characterize the inflammatory cell populations in *Tg(ELa1-Lta,b)* pancreata in more detail we performed additional flow cytometry experiments, analysing CD62L, CD69 and CD44 positive cell populations (**Figure 17**). A drastic increase in the activation of memory and effector $CD4^+$ and $CD8^+$ T-cells in *Tg(ELa1-Lta,b)* pancreata was observed at the age of 12 months when compared to C57BL/6 controls.

Additionally, we found substantially enhanced recruitment of $FoxP3^+CD25^+$ regulatory T-cells (T_{reg}) in *Tg(ELa1-Lta,b)* pancreata compared to negative littermates. This phenomenon of elevated T_{reg} cells in inflamed tissues was already described in several autoimmune diseases like rheumatoid arthritis, multiple sclerosis and Crohn's disease (Campbell and Koch, 2011) (Cao et al., 2003) (Kumar et al., 2006) (Maul et al., 2005) (Uhlig et al., 2006). Furthermore, these changes could be seen in the draining lymph node of the pancreas, the mesenteric lymph node and the spleen - albeit at much lower level - suggesting that the primary locus of auto-reactive immune activation is within the pancreas.

Next, we evaluated the T-helper cell subsets (Th1/Th2/Th17) on mRNA level, based on their distinct cytokine production profile (**Figure 18**). Strong upregulation of *Ifnγ* and *Tnfα* and the transcription factor *T-bet* in 12-month-old *Tg* mice indicates the presence of predominantly Th1-type cells. Although, the expression of Gata3 is known to control the development of Th2 lineage, recently a stable $Gata3^+$/$T\text{-}bet^+$ cell subset was described (Hegazy et al., 2010). Cytokines secreted by Th2 cells such as *Il4* and *Il10* were also slightly upregulated. Cytokines produced by Th17 cells, *Il17A*, *Il17F*, as well as some Th2 cytokines (*Il5 and Il13*), were undetectable in the pancreata of *Tg(ELa1-Lta,b)* mice compared to controls (data not shown), which correlates to our analyses of human AIP pancreata. Of note, in peripheral blood of human AIP patients, Th1 cells but not Th17 cells are predominant over Th2-type cells (Okazaki et al., 2010).

To macroscopically identify organised inflammatory follicles within the pancreata of *Tg(ELa1-Lta,b)* mice we performed immunohistochemical stainings. By the age of three months transgenic pancreata contained high endothelial venules (HEV) (**Figure 19**). The above described follicles developed into tertiary lymphoid organs (TLOs) characterized by the presence of distinct T- and B-cell areas containing clusters of proliferating lymphocytes, $CD21/35^+$ germinal center B-cells as well as $FDC\text{-}M1^+$ follicular dendritic cell networks.

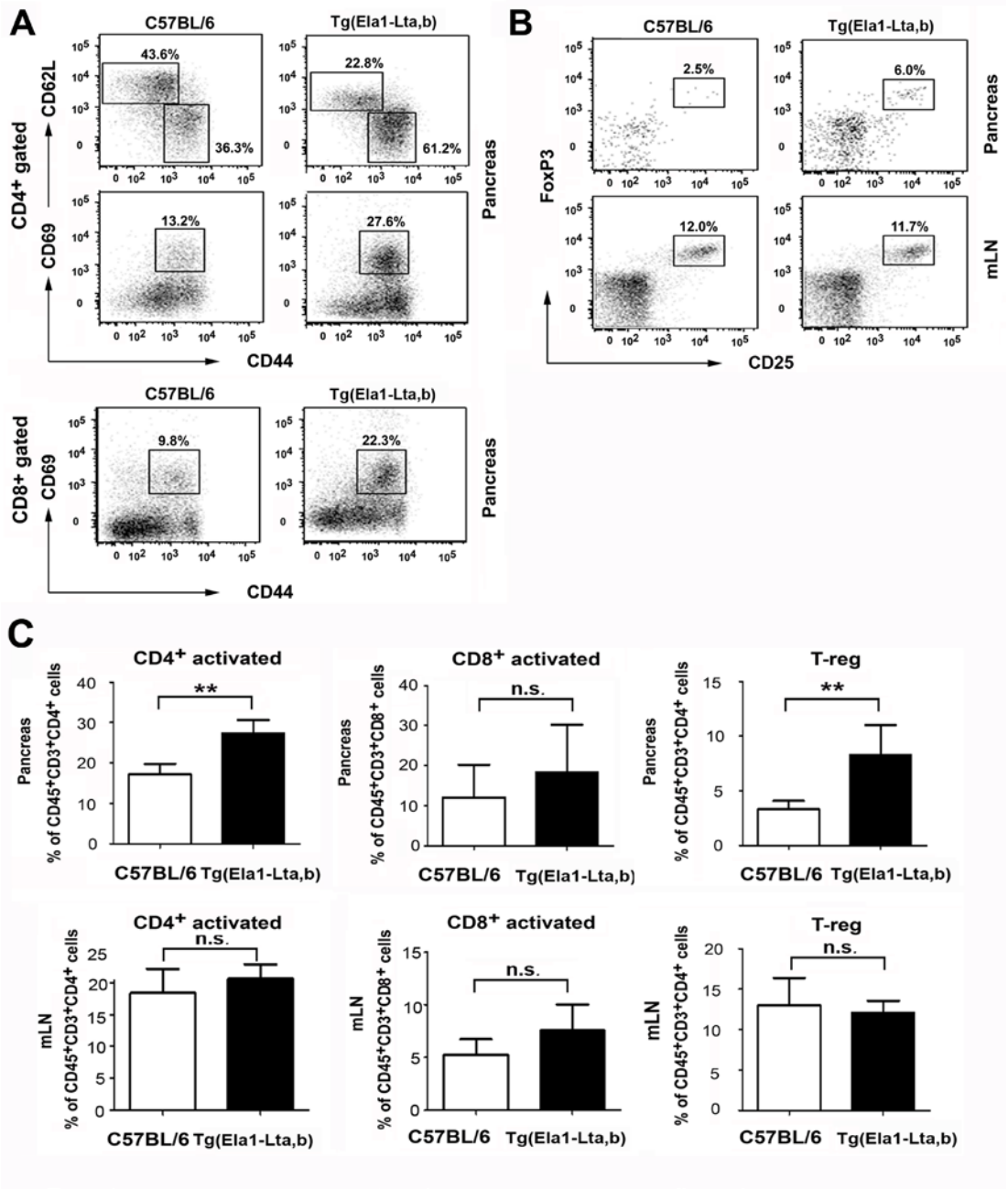


Figure 17. Flow cytometry of chronically inflamed *Tg(ELa1-Lta,b)* pancreata. (A) Gating strategy for activated $CD4^+$, $CD8^+$ T cells as well as **(B)** regulatory T cells ($FoxP3^+CD4^+$) in pancreata and mesenteric lymph nodes (mLN) of 12 month-old *Tg(ELa1-Lta,b)* and C57BL/6 control mice. **(C)** Quantification of activated $CD4^+$, $CD8^+$ T-cells and regulatory T-cells in pancreata and mesenteric lymph nodes (mLN)) of 12 month-old *Tg(ELa1-Lta,b)* and C57BL/6 control mice.

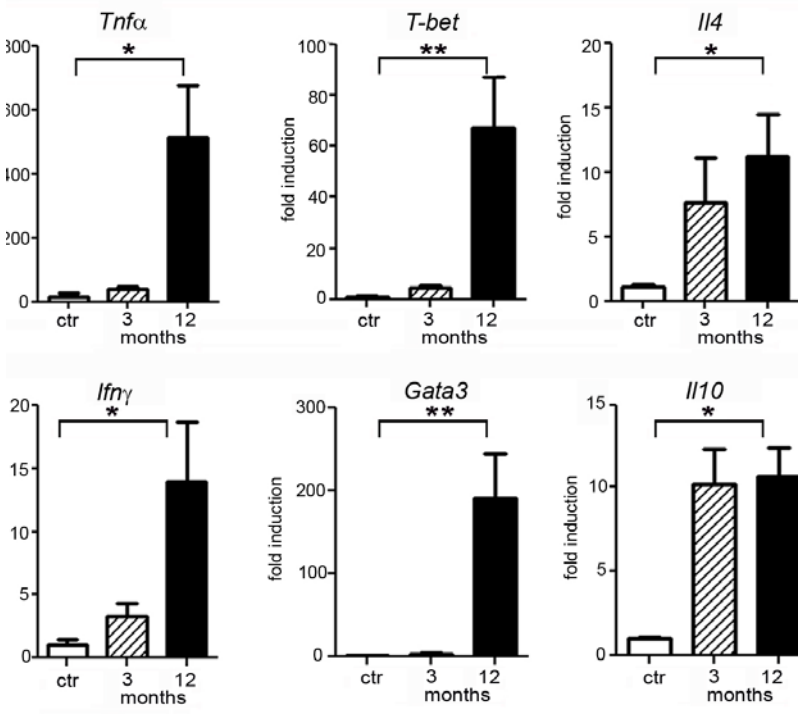


Figure 18. mRNA transcript analysis of T-helper cell subsets. Quantitative RT-PCR analysis of pancreatic *Tnfα, Ifnγ, T-bet, Gata3, Il4 and Il10* transcripts in 3 and 6 month-old *Tg(ELa1-Lta,b)* pancreata compared to negative littermates (3 and 6 months pooled).

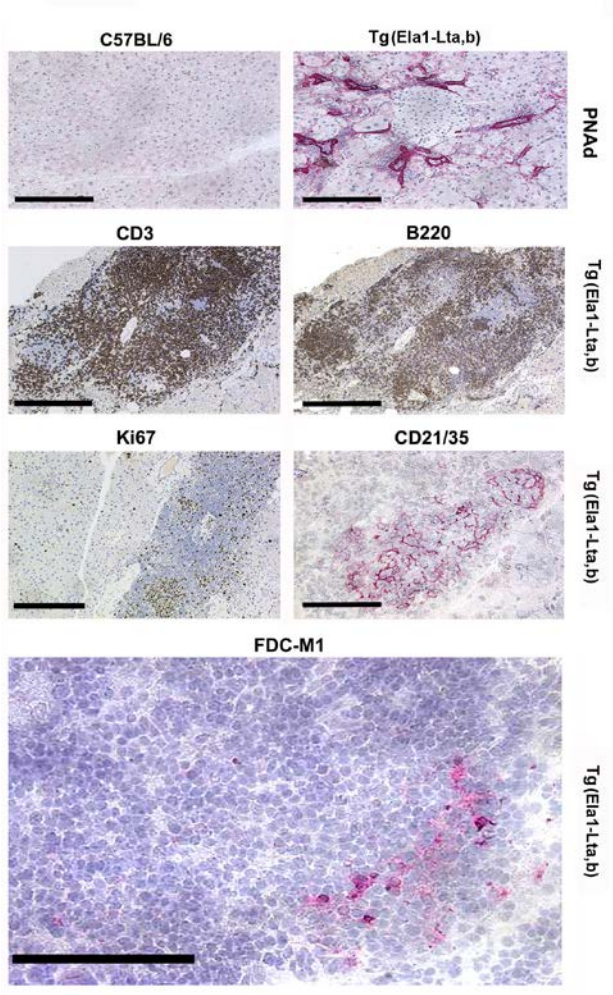


Figure 19. Characterization of tertiary lymphoid organs in *Tg*(Ela1-LTa,b) mice. Between 3 and 12 months of age *Tg(ELa1-Lta,b)* develop high endothelial venules, whereas HEVs are absent from age matched C57BL/6N mice (PNAd staining) (scale bar: 100 μm). After 6 months of age inflammatory infiltrates are found with distinct $CD3^+$ and $B220^+$ T- and B-cell compartments (scale bar: 200 μm). The cluster of $Ki67^+$ proliferating inflammatory cells (scale bar: 200 μm) is indicative of a germinal center, which is supported by the presence of $CD21/35^+$ germinal center B-cells (scale bar: 100 μm). Of note, follicular dendritic cell networks ($FDC\text{-}M1^+$) (scale bar: 100 μm) can be found in the inflammatory infiltrates of 12 months old *Tg(ELa1-Lta,b)* mice.

2.3.8. Analysis of total immunoglobulins in *Tg*(ELa1-Lta,b) and wild-type mice

TLOs with germinal centers and an auto-inflammatory, activated lymphocytic environment in the exocrine part of *Tg(ELa1-Lta,b)* pancreata are reminiscent of an autoimmune disease. Thus, we investigated whether transgenic mice display increased immunoglobulins and/or auto-antibodies.

Serum IgG4 is known to be elevated in 50% of AIP patients; however, IgG4 does not exist in mice. We therefore analyzed total IgG (including subtypes IgG1, IgG2a and 2b, IgG3), IgM and IgA levels at twelve-month-old *Tg(ELa1-Lta,b)* mice and negative littermates. We found a significant increase in total IgG (1.56 fold, *P*=0.0148), IgG1 (1.79 fold, *P*=0.0128), IgG2b (2.99 fold, *P* =0.0073), and IgM (1.65 fold, *P*=0.0050) of *Tg(ELa1-Lta,b)* mice when compared to C57BL/6 mice. The observed increase in immunoglobulins parallels the hyper-γ-globulinemia found in human disease (**Figure 20**).

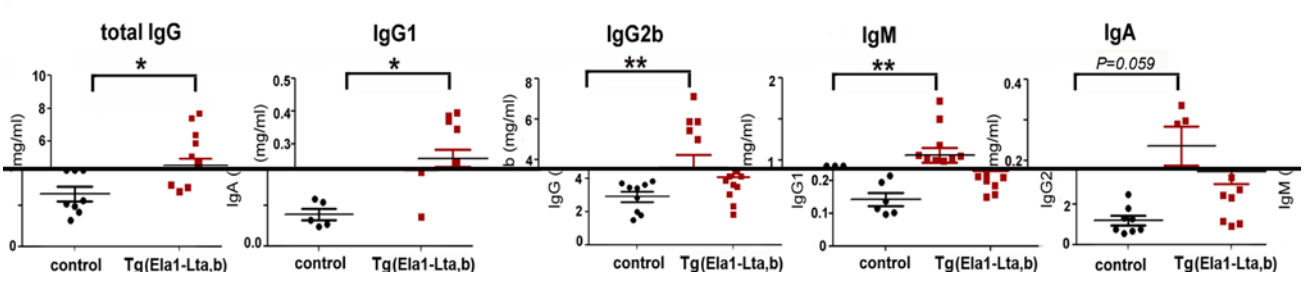


Figure 20. Serum immunoglobulin measurement. Serum analysis of 12 month-old *Tg(ELa1-Lta,b)* shows significantly elevated total IgG, IgG1, IgG2b, IgM and IgA (n.s.) levels compared to age matched negative littermates.

2.7.9. Development of auto-antibodies in transgenic mice

The increased serum immunoglobulin levels and the presence of TLOs in the pancreas suggest that *Tg(ELa1-Lta,b)* mice had developed an autoimmune phenotype. Therefore, we investigated whether anti-nuclear antibodies (ANA) are present in sera of transgenic mice. Sera from six-, twelve- and eighteen-month-old mice were subjected to an indirect immunofluorescent microscopy assay (**Figure 21**). Although we could not detect auto-antibodies at the age of six months in transgenic or wild-type mice, ANA were present in sera of *Tg(ELa1-Lta,b)* mice with an incidence of around 66% at the age of twelve months (**Table 3**). To assess the specificity of ANA, we measured plasma concentrations of anti-nucleosome and anti-dsDNA antibodies. Levels of both antibodies were increased in twelve-month-old *Tg* mice (anti-Nucleosome *P*=0.1003; anti-dsDNA: *P*=0.0440; Figure 5C). Rheumatic factor

(RF) is elevated in sera of AIP patients (Okazaki and Chiba, 2002). Similarly, transgenic mice showed an almost four-fold increase in serum RF compared to negative littermates (*P*=0.029). We also identified an increase in anti-Smith IgG, indicating systemic autoimmune disease (*P*=0.028, Figure 5C). Thus, auto-antibodies seen in human AIP, as well as indicators of systemic autoimmune disease are present in the sera of *Tg(ELa1-Lta,b)* mice.

As the inflammatory process started in the pancreas we investigated whether *Tg(ELa1-Lta,b)* mice developed auto-antibodies against pancreatic self-antigens, as observed in human AIP. (Asada et al., 2006) (Okazaki et al., 2010). Thus, we measured anti-PSTI, anti-LF and anti-Lipase antibodies in twelve-month-old *Tg* mice (Figure 5D). We found significantly elevated levels of auto-antibodies against PSTI, LF, and the Lipase in *Tg(ELa1-Lta,b)* mice (PSTI: *P*=0.0003; LF: *P*=0.036; Lipase: *P*=0.028).

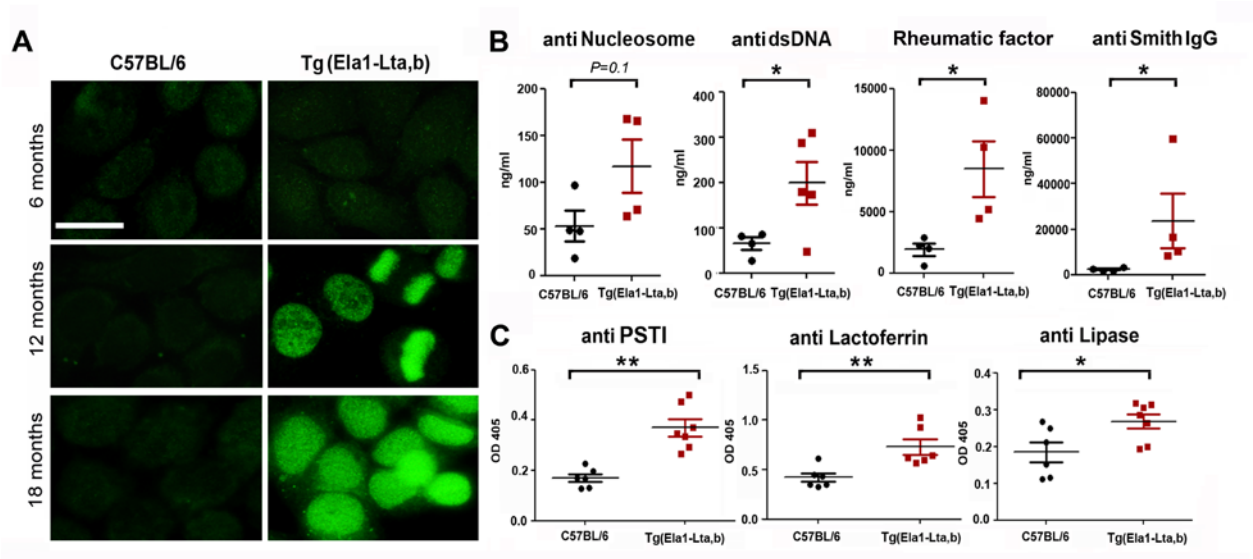


Figure 21. Detection of antinuclear antibodies (ANA) and auto-antibodies against pancreas-specific proteins. (A) Detection of antinuclear antibodies (ANA) on Hep-2 cells with sera obtained from 6, 12 and 18 month-old C57BL/6 and *Tg(ELa1-Lta,b)* mice (scale bar: 20μm). **(B)** Transgenic mice possess elevated serum anti nucleosomes and anti dsDNA antibodies, as well as Rheumatic factor and anti-Smith IgG levels which are indicative of a systemic autoimmune disease. **(C)** Detection of anti-PSTI, anti-Lactoferrin and anti-Lipase antibodies in sera from 12 months old C57BL/6 and *Tg(ELa1-Lta,b)* mice.

	C57BL/6			Tg(Ela1-Lta,b) #58			Tg(Ela1-Lta,b) #40		
	% ANA positive	ANA positive (mice)	Total (n)	% ANA positive	ANA positive (mice)	Total (n)	% ANA positive	ANA positive (mice)	Total (n)
6 months	0%	0	5	0%	0	5	20.00%	1	5
12 months	0%	0	5	66.66%	12	18	100%	5	5
18 months	0%	0	5	75%	5	7	75%	5	7

Table 3. Incidence of AIP in *Tg(ELa1-Lta,b)* transgenic lines #58 and #40. Incidence and percentage of ANA is indicated for both lines and C57BL/6 mice.

2.3.10. *Tg*(ELa1-Lta,b) T-cells cause acute pancreatic damage in Rag1$^{-/-}$ mice

To examine the immune cells contributing to pancreatic damage in AIP pathogenesis, we transferred C57BL/6 and *Tg* splenocytes into *Rag1*$^{-/-}$ recipients. 7 days post splenocyte transfer *Tg* donor CD3^{+} cells, but not *Tg* donor B220^{+} B-cells (data not shown) were found in pancreata of *Rag1*$^{-/-}$ recipient mice. Concomitantly, significant elevation of serum amylase levels was observed (**Figure 22**). In contrast, adoptive transfer of C57BL/6 splenocytes neither led to influx of immune cells into the pancreas nor to detectable pancreatic damage. These experiments suggest that *Tg* T-cells are capable of damaging acinar cells in *Tg(ELa1-Lta,b)* mice.

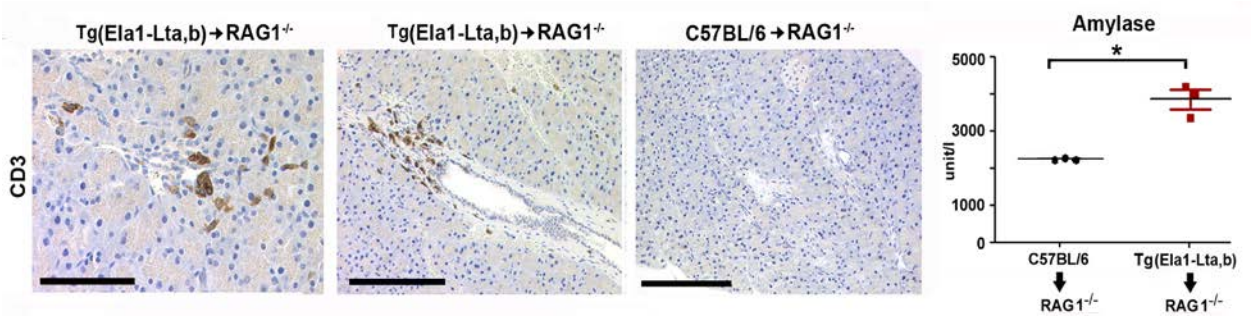


Figure 22. Representative pathological findings from *Rag1*$^{-/-}$ pancreata after spleen cell transfer with cells from 12 months old *Tg(ELa1-Lta,b)* and C57BL/6N mice. Presence of CD3^{+} T cells in *Rag1*$^{-/-}$ tissue indicates that the autoimmune disease is transferable (scale bars from left to right: 100 μm, 200 μm, 200 μm). The transferred immune cells from *Tg(ELa1-Lta,b)* mice into *Rag1*$^{-/-}$ pancreas led to pancreatic tissue damage shown by elevated amylase values compared to cells from C57BL/6 mice.

2.3.11. Glomerulonephritis in *Tg*(ELa1-Lta,b) mice

Extra-pancreatic pathologies are recognized as important manifestations of IgG4-related systemic disorders during AIP. Since increased immunoglobulin levels were found in sera of *Tg(ELa1-Lta,b)* mice, we investigated possible involvement of other organs. Indeed,

transgenic mice developed glomerular lesions, which became apparent at the age of twelve months and were more prominent after eighteen months of age. The mesangial matrix of *Tg(ELa1-Lta,b)* kidneys was broadened, resulting in narrowed peripheral capillaries as demonstrated by light- and electron microscopy. Immunohistochemistry demonstrated IgG deposits in the mesangium, but only occasionally in peripheral capillary walls. Quantification of immune deposits showed a significant increase after one year of age (**Figure 23**).

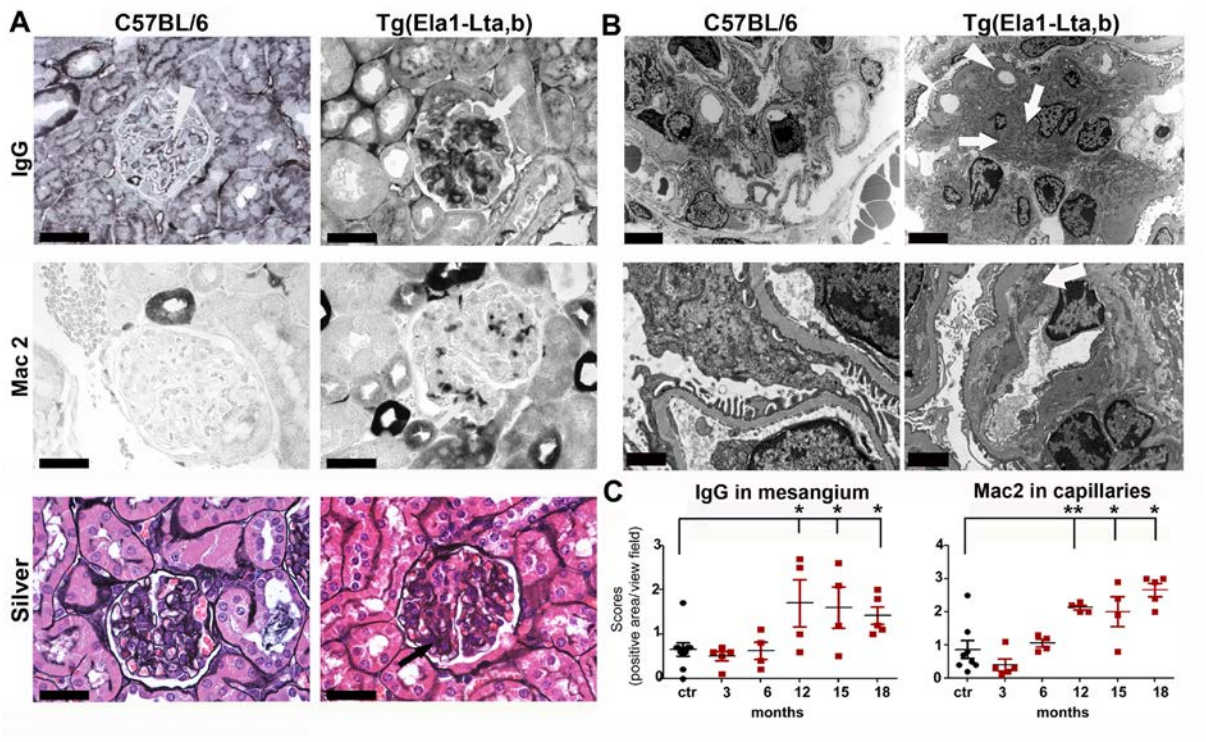


Figure 23. Extrapancreatic manifestation of AIP in *Tg(*ELa1-Lta,b) mice. (A) Representative pathological findings in kidneys of *Tg(*Ela1-LTa,b) mice. Prominent IgG deposits, indicated with white arrow in the mesangium of *Tg(*Ela1-LTa,b) kidneys compared to age matched C57BL/6 controls. Accumulation of $Mac2^+$ macrophages is observed in the capillaries of transgenic mice but not in negative littermates. The silverstain illustrates accumulation of mesangial matrix (black arrow), and the reduced number of open glomerular capillaries as compared to the control (scale bar: 20 μm). **(B)** Electronmicroscopy demonstrates mesangial matrix expansion (white arrows) and narrowing peripheral capillaries (white arrow heads) in transgenic mice compared to C57BL/6 mice. Thickening of the capillary wall and narrowing of the glomerular capillary by cellular interposition is illustrated in the lower right panel (white arrow) (scale bar: 5μm in upper row, 1 μm in C57BL/6 and 2 μm in *Tg(ELa1-Lta,b)* mice, lower row). **(C)** Quantification of IgG deposits in mesangium and macrophage accumulation in capillaries in 3, 6, 12, 15 and 18 months old *Tg(*Ela1-LTa,b) (n=4-5) compared to a pool (n=8) of all representative age groups from C57BL/6 mice. *: $P>0.05$; **: $P<0.01$, ***: $P<0.001$.

Increased accumulation of $Mac\text{-}2^+$ macrophages in glomeruli correlated with age. Electron microscopy confirmed the mesangial matrix expansion and some electron dense immune

deposits were found predominantly in the mesangium. Occasionally interposition of cellular protrusions into the wall of peripheral capillaries was present. Podocyte foot processes were well preserved and comparable to the wild-type controls. The described renal lesions resemble an immune-complex glomerulonephritis, which is associated with AIP in some patients.

2.3.12. Autoantibodies against commensal bacteria in *Tg*(ELa1-Lta,b) animals

In wt, specific pathogen free (SPF) mice, high-affinity IgG responses specific for microbiota cannot be detected in serum (Slack et al., 2009). However, gut microbiota can contribute to autoimmune diseases, e.g. Crohn's disease and rheumatoid arthritis (Tiwana et al., 1997) (Newkirk et al., 2010).

To determine whether the observed hyper-γ-globulinaemia may be partly driven by increased systemic exposure to the intestinal microbiota, we used flow cytometry to quantify the serum titers of IgG1, IgG2b and IgM directed against the surface of live bacteria (**Figure 24**). Twelve-month-old *Tg(ELa1-Lta,b)* mice display increased IgG1, IgG2b and IgM titers against both bacterial strains when compared to wild type littermates.

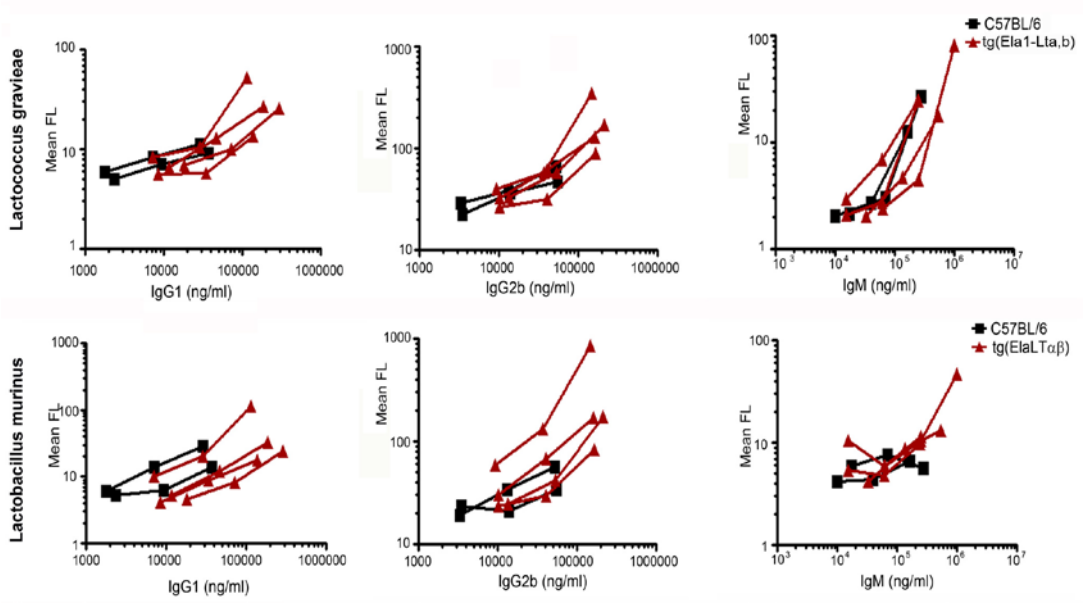


Figure 24. Autoantibodies against commensal bacteria. 12 month-old transgenic mice show an elevated serum IgG1, IgG2b and IgM titer against two of their own commensal bacterial strains (two facultative aerobic bacteria isolated from the fecal microbiota of *Tg(ELa1-Lta,b)* mice: *Lactococcus gravieae* and *Lactobacillus murinus*) compared to negative littermates. Pure cultures of the indicated bacteria were stained with dose-titrations of serum from *Tg(ELa1-Lta,b)* or C57BL/6N mice. Serum antibody coating of bacteria was visualised using monoclonal PE-conjugated anti-mouse IgG1, FITC-conjugated anti-mouse IgG2b, APC-conjugated anti-mouse IgM and quantified per bacterium by flow cyotmetry. Resulting MFI was plotted against total IgG1, IgG2b and IgM added to the assay as determined by ELISA.

The timecourse of anti-microbiota IgG appearance suggests that at least a portion of observed hyper-γ-globulinaemiea in *Tg(ELa1-Lta,b)* mice is due to intestinal mucosal damage resulting in an increased systemic exposure to gut bacteria.

2.3.13. The role of monocytes and lymphocytes in the initiation and progression of AIP

To define the cellular and molecular mechanisms of AIP development we crossed *Tg(ELa1-Lta,b)* mice to mice lacking B- and T-lymphocytes (*Rag1*$^{-/-}$) or pro-inflammatory monocytes (*Ccr2*$^{-/-}$) (Mildner et al., 2009) (Kuziel et al., 1997).

Crossing to *Rag1*$^{-/-}$ mice resulted in early pancreatic injury, based on serum lipase levels (similar to *Tg(ELa1-Lta,b)* mice) and acinar to duct metaplasia formation (**Figure 25B**). Furthermore *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ mice showed increased F4/80^{+} macrophage infiltration. On the contrary, lack of pro-inflammatory monocytes rescued the early onset of pancreatic tissue damage and concomitant lipase elevation; however, modest lymphocytic infiltration was observed. This suggests that proinflammatory monocytes support the initiation of pancreatic tissue damage in transgenic mice (**Figures 25A and B**).

Altered transcriptional expression at 8 weeks corroborated this difference. *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ mice displayed an increase in homeostatic chemokines, while in the absence of B and T-cells, genes involved in macrophage activation, e.g. *Tnfα*, *Il6* and *Ccl2* are significantly increased (**Figure 25C**). Between three and six months, B and T cells also seem to contribute to pancreatic inflammation, as serum lipase levels show similarly reduced levels in both *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ and *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ mice (**Figure 25B**). At later time points (twelve months), chronic tissue damage appeared to be driven by lymphocytes, as *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ show organized T and B-cell infiltrates concomitant with significantly elevated serum lipase levels compared to *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ mice. In contrast to *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$, 12 months old *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ mice displayed elevated anti-PSTI antibody levels (**Figure 26**), and immune deposits in the kidneys (**Figure 27**). Thus, lack of pro-inflammatory monocytes protects from early pancreatic damage, but it does not affect the extent of AIP. From three months onwards, lack of lymphocytes resulted in reduced tissue damage and failed to induce AIP, suggesting that lymphocytes are directly involved in sustaining tissue damage and AIP in *Tg(ELa1-Lta,b)* mice.

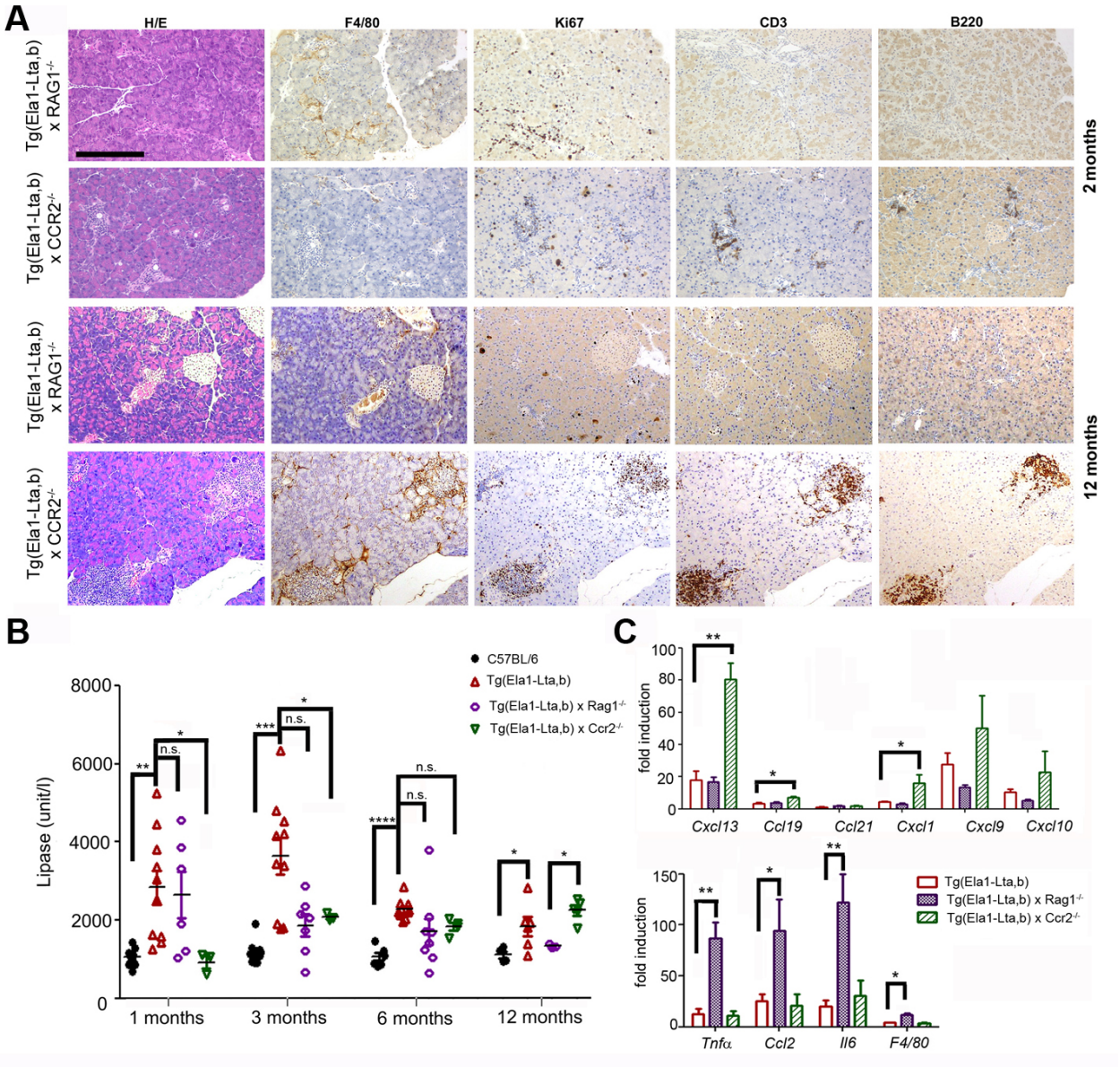


Figure 25. CCR2^{+} macrophages aggravate the initiation phase of chronic pancreatitis whereas lymphocytes contribute to late damage and autoimmunity. (A) Immunohistochemical characterization of 2 and 12-month-old *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ and *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ mice. H/E, F4/80^{+} macrophages, Ki67^{+} proliferating inflammatory cells and acinar cells, CD3^{+} T-cells and B220^{+} B-cells are indicated (scale bar: 200 μm). **(B)** Serum lipase levels of 1, 3, 6 and 12 month-old C57BL/6; *Tg(ELa1-Lta,b)*; *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$; *Tg(ELa1-Lta,b)* x *Ccr2*$^{-/-}$ mice. **(C)** Quantitative real-time PCR results from 8 weeks old mice show difference of pro-inflammatory chemokines (CXCL1, CXCL9, CXCL10) and genes involved in macrophage activation (TNFα, MCP-1, IL-6). Data are shown in fold changes.

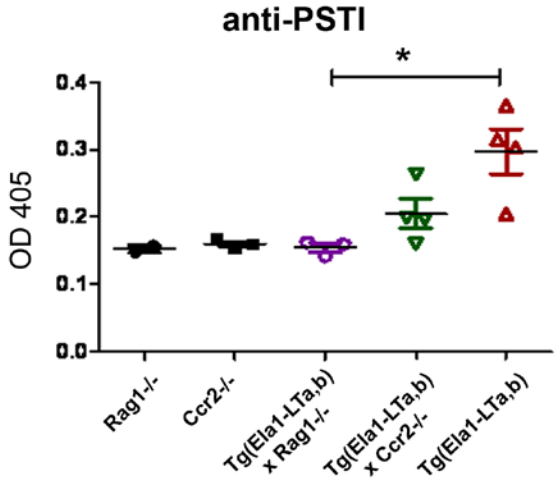


Figure 26. Detection of auto-antibodies in serum transgenic mice. Lack of proinflammatory monocytes could not rescue the development of anti-PSTI antibodies in *Tg(Ela1-LTa,b)* mice.

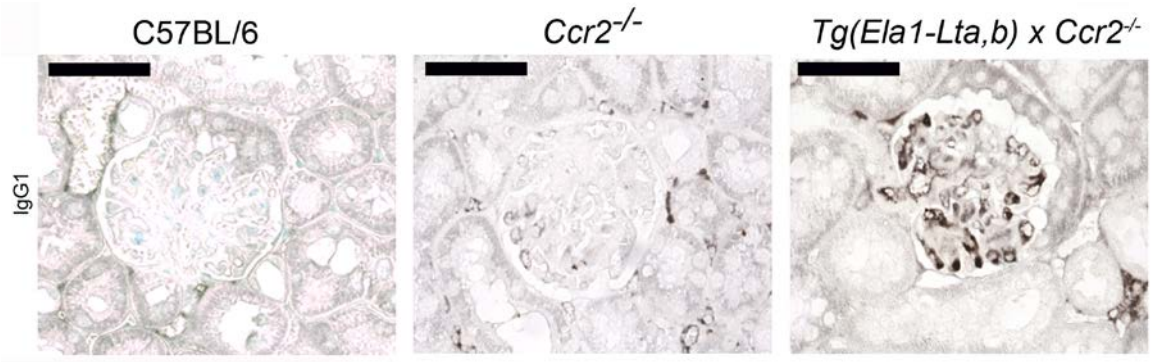


Figure 27. Detection of renal immune complexes in *Tg(Ela1-LTa,b) x Ccr2*$^{-/-}$ mice. IgG deposits in kidneys from mice of various genotypes illustrates prominent IgG1 deposits in *Tg(ELa1-Lta,b)* x *CCR2*$^{-/-}$ mice. Scale bar 50µm. In contrast, *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ lack glomerular IgG1 deposits (data not shown).

2.3.14. Corticosteroid treatment is anti-inflammatory but not anti-autoimmune in *Tg*(ELa1-Lta,b) mice

We next tested whether corticosteroid treatment, an established therapy for treating autoimmune disorders including AIP (Kamisawa et al., 2004), would be effective in *Tg(ELa1-Lta,b)* mice. Prednisolone was administered four weeks long through the drinking water to twelve-month-old transgenic and wild type mice. Indeed, prednisolone treatment in mice with established AIP significantly reduced histological signs of inflammation, the number of infiltrating B- and T-cells, macrophages and also acinar cell proliferation. The treatment effectively reduced acinar-to-duct metaplasia lesions as well (**Figures 28A and B**). However, the systemic autoimmune reaction was not affected, indicated by the persistence of auto-

antibodies (anti-PSTI) and immune complex deposition in the kidneys (**Figure 28D, E and F**). Severe side effects of long-term steroid treatment and in addition frequent relapses (approx. 40 %) are well-documented in patients (Kalaitzakis and Webster, 2011).

Therefore, we compared prednisolone treatment with inhibitors of the CD40L-CD40 or the $LT\alpha_1\beta_2$-LTβR signalling pathway. CD40 is a cytokine receptor expressed mainly on B-cells and antigen presenting cells (APCs). CD40 activation by its ligands CD40L or CD154, which is secreted by activated $CD4^+$ T lymphocytes, stimulates APCs, namely dendritic cells, monocytes and macrophages to produce TNF, IL-1β, and IL-6. This signalling is essential for the induction of affinity maturation, and class switch recombination in response to T-dependent antigen. Antibodies against CD40L were shown to prevent T_H1 mediated autoimmune diseases (e.g. with preventing expansion of germinal center B-cells or activation of monocytes) in mouse models of diabetes, SLE, RA and MS ((Mohan et al., 1995) (Balasa et al., 1997) (Durie et al., 1993). Treatment with anti-CD40L antibody in *Tg*(Ela1-LTa,b) mice failed to reduce the extent of AIP, although a decrease in serum IgG levels confirmed treatment efficacy (**Figure 29**).

In contrast, LTβR-Ig (a soluble chimeric protein that efficiently inhibits LTβR signalling) (Force et al., 1995) abrogated pancreatic inflammation as well as acinar-to-duct metaplasia upon four weeks of treatment (**Figures 28A and B**). Moreover, LTβR-Ig treatment significantly reduced the expression of chemokines and cytokines compared to prednisolone treatment (**Figure 28C**). Importantly application of LTβR-Ig also led to a remarkable reduction of auto-antibodies (e.g. against PSTI), and strongly reduced the deposition of immune complexes in the glomeruli. This suggests that in *Tg(ELa1-Lta,b)* mice, prednisolone acts rather as an anti-inflammatory than anti-autoimmune therapy (**Figures 28D-F**).

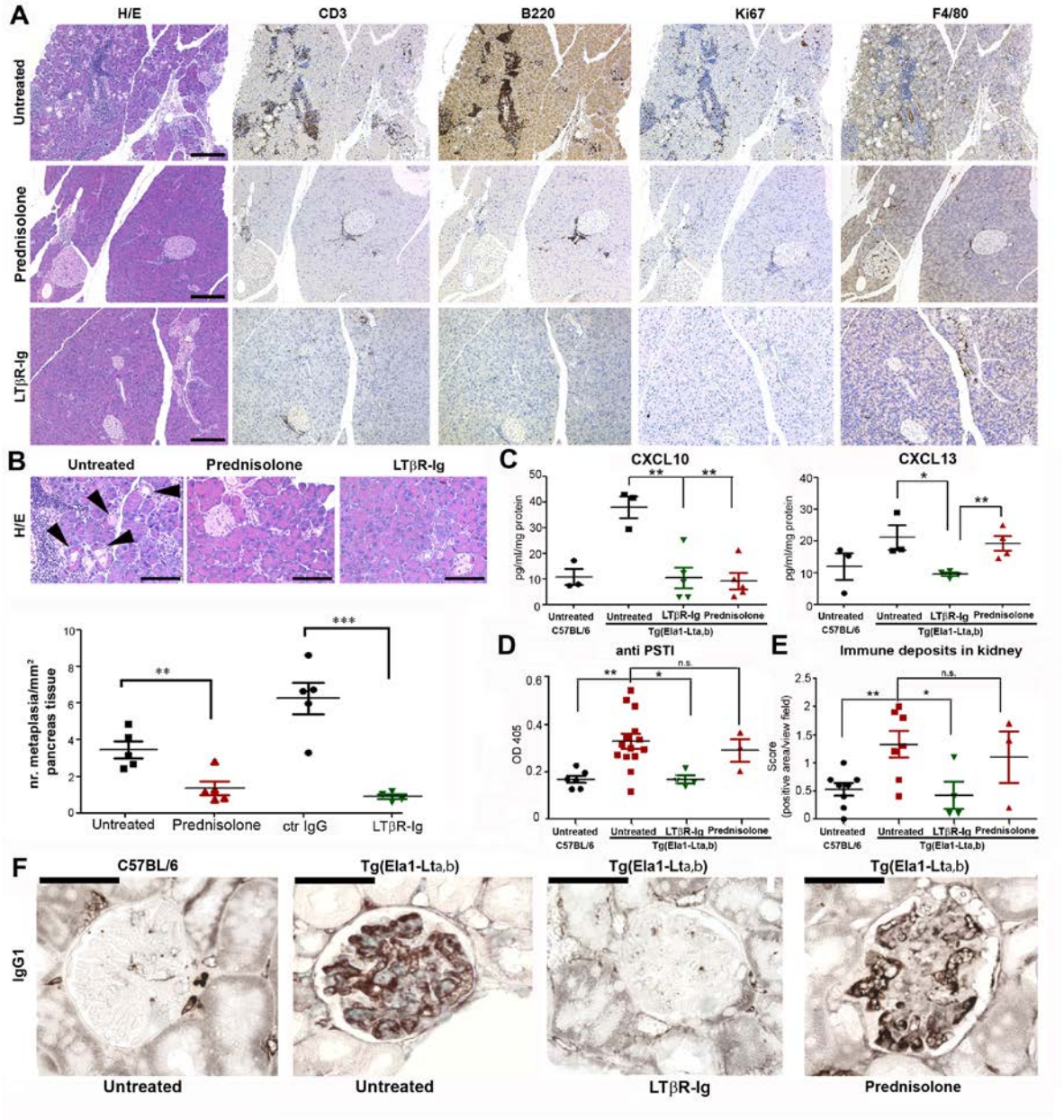


Figure 28. Corticosteroid treatment as well as blocking the LTβR signalling axis reduced the severity of inflammation. (A) Immunohistochemical characterization of pancreatic tissue from 12 months old mice treated with 10 mg/kg prednisolone (second row) and mice injected weekly with 100 μg LTβR-Ig fusion protein (third row), compared to age matched untreated *Tg(ELa1-Lta,b)* mice (first row) (scale bar: 200μm). H/E, $CD3^+$ T-cells, $B220^+$ B-cells, $Ki67^+$ proliferating inflammatory cells and acinar cells and $F4/80^+$ macrophages are indicated (scale bar: 200 μm). **(B)** Both steroid and LTβR-Ig treatment reduces the number of acinar to duct metaplasia. H/E staining of 12 months old *Tg(ELa1-Lta,b)* mice untreated, treated with corticosteroid and LTβR-Ig respectively (scale bar: 100 μm). Number of acinar to duct metaplasia was quantified and normalized to the total surface of pancreas. **(C)** Detection of CXCL10 and CXCL13 protein expression in pancreata of treated and control mice. **(D)** Detection of anti-PSTI antibodies before and after prednisolone or LTβR-Ig treatments compared to wt controls. **(E)** Quantification of IgG1 deposits in the glomeruli after four week-long treatments with either prednisolone or LTβR-Ig compared to age matched *Tg(ELa1-Lta,b)* and C57BL/6 animals. **(F)** Representative images of IgG1 deposits in kidneys after LTβR-Ig and prednisolone treatment. Scale bar: 50μm.

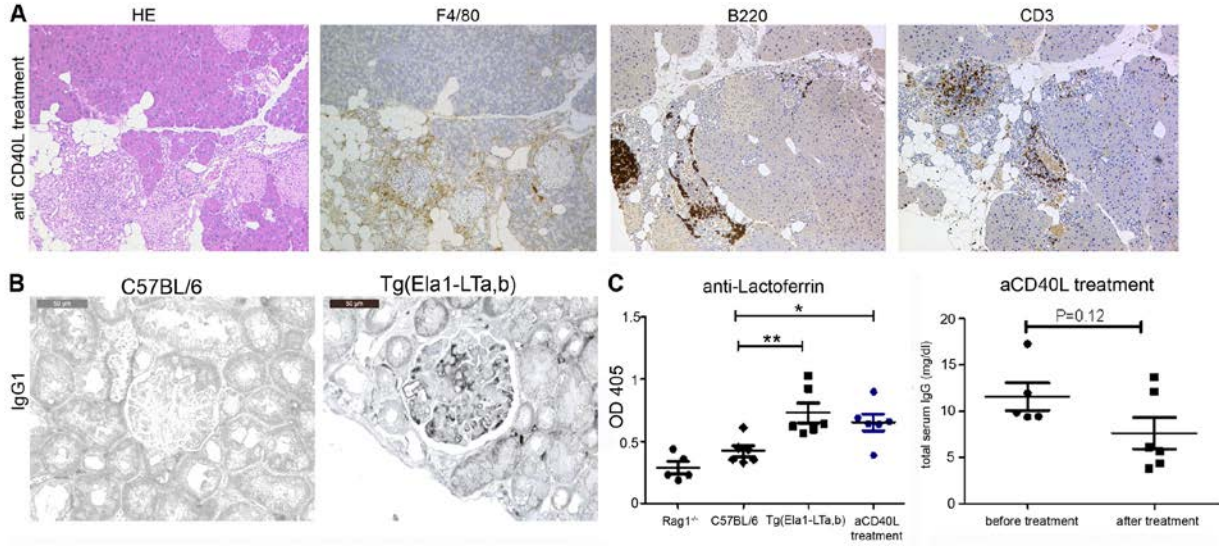


Figure 29. Anti-CD40L treatment did neither reduce pancreatic inflammation nor the level of anti-lactoferrin auto-antibodies. (A) Immunohistochemical characterization of pancreatic tissue from 12 months old mice treated with 100 μg anti-CD40L antibody for 4 weeks. $B220^+$ B-cells, $CD3^+$ T-cells, $F4/80^+$ macrophages and $Ki67^+$ proliferating inflammatory cells and acinar cells are indicated. Scale bars: 200 μm. **(B)** Visualization of IgG1 deposits in the glomeruli after 4 week-long treatment with α-CD40L antibody compared to untreated 12-month-old *Tg(ELa1-Lta,b)* and C57BL/6 animals. Scale bars: 50 μm. **(C)** Detection of anti-lactoferrin antibodies after 4 week-long treatments with aCD40L antibody compared to $Rag1^{-/-}$ controls. Comparison of total serum IgG levels before and after aCD40L treatment.

2.4. Discussion

The underlying mechanisms inducing and maintaining AIP remain elusive, thus far. Here, we show that patients suffering from AIP display high pancreatic LTαβ and chemokine expression. Of note, AIP and chronic pancreatitis with severe fibrosis each displayed unique expression signatures. Not only could we detect a strong upregulation of LT on infiltrating lymphocytes, but also on acinar cells that have undergone metaplasia.

Recently, IL17 was proposed to be crucial for autoimmune disease development as well as for TLO generation in non-lymphoid organs, stimulating stromal CXCL13 and CCL19 expression (Rangel-Moreno et al., 2011). However, we could not find significant changes in pancreatic IL17 mRNA expression in human AIP patients compared to healthy controls, nor could we find IL17 transcripts in *Tg*(Ela1-Lta,b) mice, suggesting that autoimmunity and TLO development during AIP are IL17-independent.

Existing mouse models for AIP all rely on inducing pancreatitis *via* experimental means i.e. by immunization with self-antigens, injection of pathogens (Haruta et al., 2010) or *via* adoptive transfer of auto-reactive cells or T_{regs} (Uchida et al., 2002),(Nishio et al., 2011),(Asada et al., 2010),(Meagher et al., 2008). Such models can be highly variable. Though spontaneous development of AIP has been reported previously in various genetic backgrounds prone to develop autoimmunity (Freitag et al., 2010), a genetic model of AIP with robust penetrance and phenotypic reproducibility on a C57BL/6 background was previously unavailable.

Based on our data derived from human AIP patients we have established a novel transgenic mouse model of AIP by expressing LTαβ specifically on acinar cells. This led to a transcription profile of inflammatory chemokines and cytokines (e.g. in pancreas and serum) reminiscent of human AIP causally linking ectopic LT expression in the pancreas to the development of AIP. At approximately six months of age, ectopic LT expression led to the generation of TLOs - as frequently found in AIP. Surprisingly, localized inflammation in the pancreas developed into AIP and systemic autoimmune disease over time. This coincided with a rise in pancreatic expression of endogenous *Lta* and *Ltb* transcripts, most likely produced by infiltrating immune cells.

AIP in *Tg* mice was characterized by (1) the development of antibodies against pancreatic self-antigens, (2) the presence of ANA, (3) the rise in total serum IgG, (4) antibodies against commensal bacteria as well as (5) extrapancreatic manifestation - mainly in the kidney.

Formation of pancreatic TLOs, T-cell polarization (mainly towards Th1), systemic autoimmunity and involvement of other organs (e.g. kidney) in *Tg* mice suggests a disease profile consistent with Type 1 human AIP. Importantly, the degree of *Lta* and *Ltb* transcript upregulation in transgenic pancreata (50 to 500 fold) was comparable to pancreata of human AIP patients (100 to 200 fold).

Flow cytometry analysis demonstrated a strong shift in the activation of effector and memory T-cells in pancreata of *Tg(ELa1-Lta,b)* mice. Further, as in other autoimmune diseases, an increase in T_{reg} cells in the pancreas was observed (Cao et al., 2003) (Kumar et al., 2006) (Maul et al., 2005) (Uhlig et al., 2006). TNF was shown to have the capacity to expand the functional T_{regs}, representing a negative feedback loop to counteract TNF-induced pro-inflammatory effects (Chen and Oppenheim, 2011). This implies that the significantly elevated TNF levels in 12-month-old transgenic mice could explain the increase in the Treg subset. However, decreased functionality of T_{regs}, or T_{eff} resistance on T_{reg}-mediated suppression has been described in various human autoimmune diseases (Long and Buckner, 2011), which could contribute to AIP development in *Tg(ELa1-Lta,b)* mice.

The autoimmune phenotype in *Tg(ELa1-Lta,b)* mice could be prevented by genetic depletion of B- and T-lymphocytes. In contrast, absence of pro-inflammatory (Ly-$6C^{hi}$) monocytes due to CCR2-deficiency pinpointed these cells to be important in initiating early acinar cell damage. However, lack of these $Ly6C^{hi}$ cells did not ablate development of autoimmunity which is in contrast to autoimmune models of the CNS (Mildner et al., 2009). Of note, adoptive transfer of transgenic splenocytes into *Rag1*$^{-/-}$ recipients resulted in the influx of T-cells, but not B-cells in the pancreata in parallel with acute pancreatic damage. These data suggest that, in contrast to the early pancreatic damage mediated by innate immune cells (e.g. pro-inflammatory monocytes), subsequent damage in *Tg(ELa1-Lta,b)* mice is mediated by cells of the adaptive immune system, particularly T-cells. This is corroborated by the strong reduction of pancreatic tissue damage (e.g. serum lipase) in *Tg(ELa1-Lta,b)* x *Rag1*$^{-/-}$ mice between 6-12 months of age. In contrast, *Tg(ELa1-Lta,b)* x *CCR2*$^{-/-}$ mice display a similar amount of pancreatic tissue damage when compared to *Tg(ELa1-Lta,b)* mice within the same time-frame.

Thus, acinar cell-specific expression of LT leads to two independent reactions: **(1)** Early onset of pancreatitis attracting innate immune cells (e.g. pro-inflammatory monocytes) and activating tissue macrophages, presumably to remove non-viable pancreatic cells. **(2)** Recruitment of lymphocytes to the pancreas results in further increase in cytokine and chemokine expression contributing to an inflammatory milieu sustaining pancreatitis,

metaplasia and enabling germinal center and TLO formation. This environment fosters activation, clonal selection and expansion of auto-reactive lymphocytes - as corroborated by antibodies against pancreatic self-antigens found in *Tg(ELa1-Lta,b)* mice.

Currently, treatment options for AIP are limited. AIP patients respond well to corticosteroid treatment; however disease relapses upon treatment discontinuation are very frequent. Interestingly, neither pro-inflammatory cytokines nor homeostatic chemokines were reduced upon steroid treatment in sera of human AIP patients, which is likely responsible for the disease relapse. Therefore, a complementary treatment which could additionally down-regulate inflammatory mediators is very likely to benefit patients.

Tg(ELa1-Lta,b) mice were also responsive to corticosteroid treatment, which failed to revert the autoimmune repertoire (e.g. anti-PSTI antibodies). In contrast, treatment with LTβR-Ig led to a strong decrease of inflammation and also ablated AIP in *Tg* mice (e.g. reduction in auto-antibodies, abrogation of renal immune deposits and decrease of pancreatic CXCL13). We also depleted germinal center B-cells using a CD40L antibody (Heikenwalder et al., 2007). Although IgG levels were reduced, the load of pancreatic inflammation and the course of AIP remained unchanged upon treatment in *Tg(ELa1-Lta,b)* mice. This could possibly be explained by the presence of long-lived plasma cells in the pancreas that do not respond to anti-CD40L treatment.

This indicates that corticosteroid treatment acts as an anti-inflammatory, whereas LTβR-Ig treatment has additional anti-autoimmune effects. Surprisingly, we also found that suppression of LTβR-signalling in mice with established AIP protects against peripheral pathologies caused by AIP (e.g. immune-complex glomerulonephritis). Therefore, inhibition of the LTβR-signalling pathway could be a viable alternative or supplementary approach for the treatment of AIP.

In summary, increased local LT expression in the pancreas is sufficient to drive chronic pancreatitis, to reproduce various clinical features of human AIP and to induce systemic autoimmunity. *Tg(ELa1-Lta,b)* mice will facilitate the study of AIP-specific pathogenic mechanisms and allow testing potential treatment interventions.

2.5. Outlook

Human AIP is believed to be a T-cell mediated autoimmune disease. In *Tg(ELa1-Lta,b)* mouse model we could show with adoptive transfer of splenocytes into $Rag1^{-/-}$ recipients, that T-cells can directly damage acinar cells.

(1) Based on this we would first like to analyse if the $CD3^+$ T-cells we found infiltrating the *Rag*$1^{-/-}$ pancreata from *Tg* donor cells, belong to the group of $CD4^+$ or $CD8^+$ T-cells. Further, we plan to investigate the mechanisms of the caused acinar cell damage with a cytotoxic T-lymphocyte killing assay. Although, isolating and culturing viable immune cells from the pancreas is experimentally difficult, a trend towards the activation of $CD8^+$ lymphocytes in the mesenteric lymph nodes was observed.

(2) Secondly, to delineate the role of B-cells in the pathogenesis of AIP and in acinar cell damage in *Tg(ELa1-Lta,b)* mice, I already initiated an intercrossing with $J_H^{-/-}$ mice (targeted deletion of the gene responsible for immunoglobulin heavy chain production provides a mouse model devoid of mature B-cells). I plan to monitor the changes in serum amylase and lipase, and follow up the composition and number of infiltrating immune cells on histology, as well as investigate the polarization of Th-cell subsets over time. Since J_H mice lack both surface immunoglobulins and secreted IgG and IgM, we cannot use the conventional ANA assay and the above described anti-PSTI or anti-LF Elisa systems to assess the role of B-cells in autoimmunity. Therefore, I plan to deplete B-cells with anti-murine CD20 (rituximab) antibody from *Tg(ELa1-Lta,b)* mice with established AIP and analyse auto-antibody production, cytokine and chemokine expression and renal immune-complex deposition.

(3) Our results indicate, that in *Tg(ELa1-Lta,b)* mice $CD4^+$ T-cell polarization mainly occurs towards Th1 phenotype, based on elevated Tnfα, Ifnγ and Tbet levels. Therefore, I would like to test the efficacy of an anti-TNF treatment in *Tg* mice. The activity of TNF can be inhibited *in vivo* by injection of a recombinant fusion protein consisting of the mouse p55 TNFR-IgG.

(4) There is now emerging evidence implicating gut microbiota in the starting phase of human autoimmune diseases. Besides inflammatory bowel disease, in which bacteria may act on local tissue directly as well as indirectly, inflammatory diseases with remote tissues affected seem to be modulated by the gut environment (e.g. in rheumatoid arthritis, type 1 diabetes mellitus or in the animal model of MS (EAE) (Berer et al., 2011). Interestingly, *Tg(ELa1-Lta,b)* mice develops auto-antibodies against their own commensal bacteria. Thus, I would like to assess whether modification of the commensal microflora would influence the induction and development of AIP in *Tg(ELa1-Lta,b)* mice. *Tg* mice will be treated with an

antibiotic cocktail, that has been shown to effectively reduce the gut intestinal bacterial burden (Ochoa-Reparaz et al., 2009). Alternatively, although cumbersome it would be feasible to generate germ free *Tg* mice to thoroughly investigate the role of commensal microflora in autoimmune pancreatitis.

2.6. Material and Methods

Human samples: Human pancreas biopsies and serum samples were obtained from the University Hospitals Zurich and Bern, the University of Graz and Ehime University. All samples were registered in the respective biobanks and kept anonymous. The research project was authorized by the Ethics Committees of the University Hospital Zurich and the Canton of Zurich (Ref. Nr.StV 26-2005), local ethics committee Bern, Graz (Ref. Nr 20-492 ex 08/09), and the Ehime University Graduate School of Medicine (#1001003).The study protocol was in accordance with the ethical guidelines of the Helsinki declaration.

PCR specific for *Tg(ELa1-Lta,b)* mice: For transgenic LTa the following primers were used: Forward primer: (Prp 5'): 5'-CTG AGT ATA TTT CAG AAC TG-3'. Reverse primer: (LTa rev): 5'-CAG AGA AAA CCA CCT GGG AG-3'. For transgenic LTb the following primers were used: Forward primer (Prp 5'): 5'-CTG AGT ATA TTT CAG AAC TG-3'. Reverse primer: (LTb rev): 5'- GAG TCT CTG AGA GGC TAG AG-3'. The following PCR conditions were established on a Gene Amp® PCR System 9700 PCR machine (Applied Biosystems): 95°C 60 sec denaturation; 55°C 50 sec annealing; 72°C 50 sec elongation; 35 cycles.

Real-time polymerase chain reaction: For mRNA expression analysis Real-time PCR was performed using Fast Start SYBR Green Master Rox (Roche) or specific TaqMan® Gene Expression assays (Applied Biosystems, AB). Primers were custom made by Microsynth or purchased from ABI. (Primer sequences and probes are listed in supplemental table 2) Real-time PCR was performed: *(1)* On an ABI PRISM 7900 HT Fast Real-Time PCR System (AB). Data were generated and analyzed using SDS 2.4 and RQ manager 1.2 software. The mRNA expression levels were normalized to the housekeeping gene *Gapdh*, primer sequences used are listed in the supplemental material. Identical cryo-blocks used for mRNA-expression analysis of human samples were controlled on histological level by Hematoxilin-Eosin (H/E) staining to verify the respective pancreatic pathology. Specimens that did not meet the respective pathological criteria were not included. *(2)* Real-time PCR was run on a Taqman 7000 or 7500 FAST (Applied Biosystems, Switzerland) under standard conditions. Transcript levels were quantified using 18S rRNA (TaqMan® Ribosomal RNA Control Reagents, Applied Biosystems) as a reference and normalized.

Flow cytometry: Pancreata from *Tg(ELa1-Lta,b)* and wt mice were harvested immediately after sacrifice and dissected free of contaminating lymph nodes or fat pads. Pancreata from two mice were pooled for the analysis. Tissues were macerated with scalpels in a petri dish and transferred in a 2 ml tube containing 2 ml Collagenase D solution (Roche 2 mg/ml) for 30 minutes at 37°C with agitation. DNase (2.5 mg/ml) was added and the tissue was drawn through an 18-gauge needle several times. After washing, lymphocytes were isolated using density gradient separation (Lymphoprep) and centrifugation at 1200 rpm for 45 minutes at room temperature. The buffy coat, containing lymphocytes was removed and washed. Cells were resuspended in FACS buffer and cell populations were characterized with antibodies against CD45, CD4, CD8, CD11b, B220, Ly6G and Ly6C.

Antinuclear antibody (ANA) detection: Sera obtained from *Tg(ELa1-Lta,b)* transgenic mice and C57BL/6 mice at the age of 6, 12 and 18 months were used to detect the presence of ANA using Hep2 cells (Euroimmun Lübeck, Germany). The cells were incubated with 1:60 and 1:200 dilutions of each mouse serum sample in PBS, followed by incubation with FITC-conjugated polyclonal goat antibody to mouse IgG-H&L (ab6785, Abcam, Cambridge, UK). The reaction was visualized using a LEICA DLMB immunofluorescence microscope equipped with FITC (absorption, 485–520 nm; emission, 520–560 nm wavelengths) filter sets. Staining intensity was determined visually based on independent observation of two experienced technicians of the routine diagnostic immunology laboratory.

Serum Anti-LF, anti-lipase and anti-PSTI Antibodies: Microtiter plates (MaxiSorp; Nalge NUNC, Roskilde, Denmark) were coated with 20 mg/ml of LF (Sigma Chemicals), 10 mg/ml PSTI (Graf et al., 2002) and 10mg/ml purified pancreatic rat lipase in 50-mM sodium carbonate buffer (pH 9.5), and kept overnight at 4^0C. The coated wells were washed with PBS and blocked with 5% nonfat milk in PBS for 2 h at 37^0C. After three washes with PBS, each well was incubated with of mouse serum sample diluted at 1:10 with PBS containing 2% nonfat milk for 2 h at room temperature. The wells were then washed with PBS and incubated with Phosphatase-conjugated goat anti-mouse antibodies (Biosource) for 1 h at room temperature. After three washes with PBS, the bound antibodies were detected with Phosphatase substrate (Dako, Glostrop, Denmark). The plates were read at 405 nm using an ELISA plate reader (Vmax, Molecular Devices, Tokyo, Japan).

Statistical analyses and software: GraphpadPrism version 5 (LaJolla, Ca) was used to construct figures and diagrams. One-way ANOVA or unpaired *t*-tests and Mann-Whithey tests were used where appropriate. Differences were considered statistically significant if $P < 0.05$ and marked with an asterisk. Asterisks indicate: *: $P<0.05$; **: $P<0.01$, ***: $P<0.001$.

Animal husbandry and samples: Animals were maintained under specific pathogen–free conditions, and experiments were approved and conform to the guidelines of the Swiss Animal Protection Law, Veterinary office, Canton Zurich. C57BL/6 and $Rag1^{-/-}$ mice were purchased from The Jackson Laboratory, $CCr2^{-/-}$ mice were purchased from the Jackson laboratory (Kuziel et al., 1997).

Generation of *Tg(ELa1-Lta,b)* mice: The promoters from Alb-LTa (Heikenwalder et al., 2005) and lck-LTb (Heikenwalder et al., 2008) expression constructs were cut out by Not I, BamH I double digest and the remaining DNA expression vector cassettes purified (LTa-expression cassette; LTb expression cassette). The modified rat pancreatic elastase promoter plasmid (deleted for the restriction site BamH I) (Swift et al., 1984) was used as template to amplify the promoter sequence. The fwd primer was designed with a 24 base overhang to create a BamH I cut site. The PCR products were inserted to pCR®2.1-TOPO® Vector by using TOPO TA cloning Kits (Invitrogen). The positive clones were selected and analyzed by sequencing (Microsynth). A clone containing the correct elastase promoter sequence was double digested by Not I and BamH I in order to isolate the elastase promoter and was then purified by using GenElute™ HP Plasmid Midiprep Kit (Sigma). The elastase promoter - Not I and BamH I fragment was then ligated into the LTa and LTb expression cassettes using a T4 DNA Ligase (New England Biolabs). Bacterial colonies containing the elastase-LTa and elastase-LTb constructs were then picked, cultivated and screened by digestion with BamHI and NotI. After sequencing plasmids, and subsequent linearization microinjection was performed into pronuclei of C57BL/6N zygotes with equimolar ratio of independent, linearized constructs harbouring each the *Lta* and *Ltb* murine cDNA.

Upon PCR analysis, from 50 born offspring, 8 were identified as single or double transgenic. Some of these potential founder lines expressed the transgene(s) on mRNA level in the pancreas (Supplemental Figures 3B, C and 4A). After expansion of those transgenic lines and further analysis of the F2 and F3 generations we selected two double transgenic, LTab-expressing lines C57BL/6N-*Tg(Ela1-Lta,b)228, 229Biat* (*Tg*228 and *Tg229* denoted as line #40 and #58) for further experiments described. Litter size and average weight (2-18 months)

remained normal in both transgenic lines when compared to age matched C57BL/6N control mice. Moreover, #40 and #58 mice displayed no obvious behavioural changes and alteration of breeding efficiency when compared to non-transgenic littermates (data not shown). Transgene expression stayed stable up to 12 months of age in both lines (Supplemental Figure 4C).

RNA extraction: RNA was extracted and used for Real-time polymerase chain reaction (PCR) as described previously (Reding et al., 2006) or with a newly established method using the Precellys®24 Dual homogenizer with MagNA Lyser Green Beads from Roche Applied Science. In short, a small piece of snap frozen tissue was transferred to a tube containing beads, 650µl of Lysis buffer (Qiagen) was immediately added and the tube immediately transferred to the Precellys and homogenized once at 6000 rpm for 30 seconds. The homogenates were transferred to a Qiashredder and the RNA was extracted following the Qiagen RNeasy Mini Kit extraction protocol with an on column DNase digestion step. Purified RNA was reversely transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. For the 7500 FAST Real-Time PCR System, RNA was transcribed using the High Capacity cDNA Reverse Transcription Kit.

Serum markers of digestive enzymes produced by the pancreas: Samples for serum analyses were collected in serum separator tubes (BD, Microtainer, 365951), allowed to clot at room temperature and centrifuged at 10000 rpm for 6 min. The serum was transferred to a cryo-tube and stored at -80 ^{0}C until further analysis. Amylase and Lipase levels were measured using a serum multiple biochemical analyzer (Ektachem DTSCII; Johnson & Johnson Inc., Rochester, NY, USA).

Histology and Immunohistochemistry: Paraffin (2µm) sections of pancreas were stained with Hematoxylin/Eosin or various primary and secondary antibodies. Paraformaldehyde (4%) fixed and paraffin embedded tissue was incubated in Ventana buffer and staining was performed on a NEXES immuno-histochemistry robot (Ventana instruments, Switzerland) using an IVIEW DAB Detection Kit (Ventana) or on a Bond MAX (Leica) system. Antibodies against murine B220^{+} B-cells (Pharmingen; 1:400), F4/80 (Serotec, 1:50) for macrophages, CD3^{+} T-cells (clone SP7, Neomarkers; 1:300) were kindly provided by R. Zinkernagel (Odermatt et al., 1991). Meca-79 (Vector 1:1000), FDCM-1 (BD Biosciences cat. Nr. 551320 rat monoclonal 1:60), CD21/35 (BD Biosciences cat. Nr. 553817 rat monoclonal

(7G6) 1:2000), Ki67 staining proliferating cells was purchased form NeoMarkers (Code RM-9106-S; 1:200). The antibody against human LTb was kindly provided by J. Browning (Biogen Idec.) Antibodies against human CD3 (neomarkers RM—9107-So rabbit monoclonal SP7 1:300) (Ventana Roche Prodiluted) CD20 (Ventana Roche Prodiluted) Amylase (Sigma 1:1000) were used for immunofluorescent stainings. Image acquisition was performed on an Olympus BX53, equipped with an Olympus DP72 camera using cellSens software or on an Olympus SZX12, equipped with JVC digital camera (KY-F70; 3CCD) using Analysis software.

Immunohistochemistry of kidneys was performed on paraffin embedded materials as previously described (Vielhauer et al., 2009). In brief, the sections were deparaffinized, rehydrated and incubated in 3% hydrogen peroxide to block endogenous peroxidases. Endogenous biotin was blocked by the Avidin/Biotin blocking Kit (Vector, Burlingame, CA). An autoclave oven (or microwave treatment) was used for heat based antigen retrieval. Incubation with the primary antibody was performed for 1 hour or overnight. Incubation with biotinylated secondary antibodies (Vector) was followed by the ABC reagent (Vector). 3'3'Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement was used as a detection system. Consecutive sections were stained for mouse IgG (Vector), macrophages using Mac-2 (Cederlane Labs, Ontario, Canada), and smooth muscle actin (DAKO, Hamburg, Germany). Glomerular staining was quantified for IgG deposits in the mesangium, IgG deposits in peripheral glomerular capillaries, monocyte/macrophage accumulation and the expression of smooth muscle actin using semiquantitative scores (from 0-3).

Treatment with prednisolone: 12 months old *Tg(ELa1-Lta,b)* and C57BL/6 mice were treated with 10 mg/kg per day Prednisolone (Sigma-Aldrich) for four weeks. To deliver a dose of 10 mg/kg per day, 30 milligram of prednisolone was dissolved in 1ml ethanol and was added to 500 ml drinking water to give a final concentration of 60 µg/ml. Prednisolone containing water was replaced every three days as this has been reported to maintain systemic levels of prednisolone (Biondo et al., 2006). After four weeks of treatment mice were sacrificed to obtain serum and pancreatic tissues.

Treatment with LTβR-Ig: 12 months old *Tg(ELa1-Lta,b)* mice were injected intraperitoneally with 100 µg LTβR-Ig fusion protein (n=5) or with MOPC21 (control IgG)

(n=5) 4 weeks long on weekly basis. After four weeks of treatment mice were sacrificed, pancreas was resected and serum was obtained through cardiac puncture.

Treatment with anti-CD40L antibody

12 months old *Tg(ELa1-Lta,b)* mice were injected intraperitoneally with 100 g a -CD40L (MR-1) antibody (n=6) or with MOPC21 (control IgG) (n=5) four weeks long on weekly basis. After four weeks of treatment mice were sacrificed, pancreas was resected and serum was obtained through cardiac puncture.

Adoptive spleen cell transfer: 12 months old *Tg(ELa1-Lta,b)* (n=3) transgenic mice and C57BL/6 mice (n=3) were sacrificed and spleen cells were obtained. Spleen cells were filtered through a 40 μm cell strainer and 5 x 10^7 cells in 200 μl PBS were intravenously injected into *Rag1*$^{-/-}$ recipient mice (n=5). 7 days after the spleen cell transfer recipient mice were sacrificed and pancreatic tissues were examined for lymphoid cell infiltration (pathological damage). In parallel, serum amylase and lipase levels from recipient mice were analyzed.

Anti-nuclesome, anti-histone, RF, anti-Smith IgG detection: Anti-nucleosome and anti-dsDNA antibodies: NUNC maxisorp ELISA plates were pre-coated with poly-L-lysine (Trevigen, Gaithersburg, MD, USA) and PBS (ratio 1:1) for 1 hour as described (Lech et al., 2011)). In brief, ELISA plates were washed with 50mM Tris 0,14M NaCl buffer and incubated with mouse Histones (both 2μg/ml) or Nucleosome (2μg/ml) in SCC buffer overnight at 4°C. Anti-Smith antibodies: NUNC maxisorp ELISA plates were coated with Smith (Sm) antigen (Immunovision, Springdale, AR) in 0,05M carbonate-bicarbonate buffer overnight at 4°C. Rheumatoid factor: ELISA plates were coated with 10 μg/ml rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) in 0,05M carbonate-bicarbonate buffer overnight at 4°C. Serum samples were diluted 1:3 for all anti-DNA and anti-Nucleosome IgG ELISAs or 1:6 for anti-Smith and anti-RF ELISAs. C57BL/6 24-week mouse serum or antibody-poor serum (IRF-4 knock-out mice) was used as negative control. C57BL/6-lpr and MRL-lpr 24-week mouse serum was used as positive control. Specific IgGs were detected by horseradish peroxidase-conjugated goat anti-mouse IgG (Bethyl Labs, Montgomery, TX, USA).

Glucose Tolerance Test (GTT): After an overnight fast, GTT was performed by i.p. injection of 1.5 g/kg body weight of glucose (Glucosteril®, Bad Homburg, Germany). Blood samples were drawn at indicated time points and glucose levels were measured using a glucometer (Bayer Ascensia Elite XL, Leverkusen, Germany). Insulin levels were measured using Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove (IL), USA).

Electron microscopy: Tissue pieces of 1 mm^3 were fixed in 2.5% phosphate buffered glutaraldehyde, post-fixed in 1% Osmium tetroxide and embedded in Epon Epoxy resin following routine protocols. Ultra-thin sections were cut at 70 nm, fixed on grids and stained with uranyl-acetate for 10 minutes and lead citrate for 10 minutes. Grids were scanned on a transmission electron microscope (Philips CM 100) available at the Center of Microscopy and Image Analysis, University of Zurich, Switzerland.

Lymphotoxin alpha protein measurement: Microtiter plates (MaxiSorp; Nalge NUNC, Roskilde, Denmark) were coated with 5 μg/ml of anti-LTaFcm (provided by Dr. Grogan, Genentech), in 100 ml of 50-mM sodium carbonate buffer (pH 9.5), and kept overnight at 4^0C. The coated wells were washed three times with PBS +0.05% Tween and blocked with 5% PBS/BSA for 1 h at room temperature. After three washes, each well was incubated overnight at 4^0C with 100 ul LT standard dilutions (RnD Systems, recombinant TNFb) and pancreas homogenates, diluted at 1:10 with 1% PBS/BSA. The wells were then washed five times and incubated with the detection antibody (50 ng/ml biotinilated anti-mouse TNFb, RnD Systems) for 2 h at room temperature. After five washes 100 μl Avidin-HRP (eBioscience) was added to each well. The bound antibodies were detected with TMB solution (eBioscience) incubated for 15 minutes in dark. After addition of 20% sulphuric acid the plates were immediately read at 450 nm using an ELISA plate reader (Vmax, Molecular Devices, Tokyo, Japan).

Cytokine Protein array and Immunoglobulin measurement: Cytokine protein and immunglobulin levels from mouse pancreatic tissue and serum, and human serum BLC were measured using a multiplexed particle-based flow cytometric cytokine assay (Vignali et al., 2000). Human and mouse kits were purchased from BioRad (Ismaning, Germany) and R&D Systems (Oxon, UK) and Millipore (Zug, Switzerland). The procedures closely followed the manufacturer's instructions. The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus, Millipore, Zug, Switzerland) Multiplexed particle-based flow

cytometric assays. Protein concentration from pancreas homogenates were determined by BCA (explain, vendor) assay, and final cytokine concentrations were normalized to total protein concentrations.

Bacterial FACS: Bacterial FACS analysis was performed as described previously (Slack et al., 2009). Two facultative aerobic bacteria were isolated from the fecal microbiota of *Tg(ELa1-Lta,b)* mice, identified as *Lactococcus garvieae* and *Lactobacillus murinus.* Briefly, 5ml LB cultures were inoculated from single colonies of plated bacteria and cultured overnight at 37°C without shaking. 1ml of culture was gently pelleted for 3min at 7000rpm in an Eppendorf minifuge and washed 3x with sterile-filtered PBS/1%BSA/Na-Azide before resuspending at approximately 10^7 bacteria per ml. Mouse serum was diluted 1:10 in PBS/1%BSA/Na-Azide and heat-inactivated at 60°C for 30min. The serum solution was then centrifuged at 13000rpm in an Eppendorf minifuge for 10min to remove any bacteria-sized contaminants and the supernatant was used to perform serial dilutions. 25µl serum solution and 25µl bacterial suspension were then mixed and incubated for 1h at 4°C. Bacteria were washed twice before resuspending in monoclonal FITC-anti-mouse IgG2b or IgA, PE-anti-mouse IgG1 and APC-anti-mouse IgM (all BD Pharmingen). After a further hour of incubation the bacteria were washed once with PBS/1% BSA/Na-Azide and then re-suspended in 2% PFA/PBS for acquisition on FACSArray using FSc and SSc parameters in logarithmic mode. Data were analysed using FlowJo software (Treestar, USA). Total concentrations of antibody isotypes in mouse serum were determined by sandwich ELISA. Coating antibodies were goat-anti-mouse IgG1, 2b, A and M (Serotech) and detection antibodies were HRP-conjugated anti-mouse IgG, IgM or IgA (Sigma). Standards were myeloma-derived purified IgG1, IgG2b, IgM (Invitrogen) and IgA (BD Pharmingen).

3. The role of LTβR-signalling in the central nervous system in health and disease - a novel mouse model to study chronic inflammation and autoimmunity in the CNS

3.1. Introduction

3.1.1. Multiple Sclerosis

Multiple sclerosis (MS) is among the most common neurological diseases in young adults and is a leading cause of neurological disability in this age group. In northern Europe and North America, the prevalence of MS is 1 in 1000. Worldwide, an estimated number of around 1.5–2 million people are affected (Hohlfeld, 2009).

MS is a chronic inflammatory disease of the central nervous system (CNS) characterized by focal areas of demyelination, multifocal inflammation, reactive gliosis and oligodendrocyte and axonal loss. Increasing evidence supports an autoimmune pathogenesis, with myelin antigens as the most plausible targets (Magliozzi et al., 2004). Although MS is thought to be primarily mediated by autoreactive T-cells, humoral immune responses also appear to be involved (Noseworthy et al., 2000). There are four clinical forms of MS: relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) MS. Blood brain barrier damage, prominent infiltration by activated $CD4^+$ T-cells and clonotypic $CD8^+$ T-cells, the presence of macrophages with phagocytosed myelin debris, reactive astrocytes and proliferating oligodendrocytes are characteristic of acute plaques. In chronic plaques, inflammation is less pronounced and generally restricted to the rim of the plaque, which exhibits gliosis, while the hypocellular center exhibits axonal and oligodendrocyte loss, and variable demyelination (Allavena et al., 2010).

The aetiology of MS remains elusive and its pathogenesis is only partly understood; however, it is assumed that both a complex genetic background and environmental factors (possibly including infectious agents) determine the susceptibility to develop the disease. The human γ-herpes virus - Epstein–Barr virus (EBV) - has long been considered as a biologically plausible causative trigger of MS. Although, no data so far explicitly support a direct etiologic role of the virus, recent studies allow for the development of testable hypotheses as to how EBV infection potentially promotes autoimmunity and central nervous system (CNS) tissue damage

in MS (Lunemann and Munz, 2009). In addition respective results indicate that immune mechanisms play an essential role in driving the disease process (Lassmann and van Horssen, 2011).

3.1.2 Experimental autoimmune encephalomyelitis (EAE), the widely used animal model of demyelinating diseases

Autoreactive T lymphocytes appear crucial in the development of demyelinating lesions. Their contribution has been studied extensively with the experimental autoimmune encephalomyelitis (EAE) model. EAE is an inflammatory demyelinating disease induced either by the subcutaneous administration of myelin peptide antigens (e.g., myelin basic protein (MBP), proteolipid protein (PLP), myelin oligondendrocyte protein (MOG)) with Freund's adjuvant (Keegan and Noseworthy, 2002), which is called active EAE. Immunization of rodents with myelin-specific proteins or peptides, leads to the generation of encephalitogenic, myelin-reactive, $CD4^+$ effector T-cells. Encephalitogenic T-cells then mediate an immune response in the CNS with the formation of perivascular cuffs of mononuclear cells primarily in the spinal cord and cerebellum (Muller et al., 2010). In EAE, the identity of the target auto-antigen, at least in part, determines the disease phenotype and pattern of lesion distribution in the CNS. For example, immune responses to MBP or PLP induce lesions located predominantly in the spinal cord whereas immunization with MOG generates optic nerve and spinal cord lesions. The clinical phenotypes reported in EAE range from monophasic (in Lewis rats with MBP immunization) to relapsing-remitting (in SJL mice immunized with PLP or dark agouti rats immunized with MOG) or chronic/chronic progressive forms (in C57Bl/6 or NOD mice immunized with MOG) (Bettelli et al., 2003). EAE can also be induced by adoptive transfer of myelin-specific T-cells, termed passive EAE (Stromnes and Goverman, 2006). A major difference between MS and EAE is that the latter requires an external immunization step to develop, whereas in humans, the sensitization to auto-antigens is not artificially induced. Sensitization to myelin antigens in EAE typically occurs through the use of adjuvant, usually containing bacterial components highly capable of activating the innate immune system via pattern recognition receptors (Libbey and Fujinami, 2011). In EAE, the inducing antigens are known, whereas in MS, there is no unique identified antigen (Constantinescu et al., 2011).

3.1.3. Immunopathogenesis of multiple sclerosis

3.1.4. Role of the T-lymphocytes

Based on animal experiments in EAE model and observation in human multiple sclerosis, research has mostly focused on the role of $CD4^+$ T cells in disease pathogenesis. **Figure 30** depicts the prevailing T-cell centric view of MS pathogenesis.

$CD4^+$ T helper (Th) cells can be classified into subsets based on their cytokine expression (**Figure 31**). The in vivo pathogenesis of various inflammatory and autoimmune diseases as well as protective immune responses tend to be skewed towards specific Th lineages (Becher and Segal, 2011). Major emphasis has been put on the nature of Th cell polarization pattern underlying MS. Th1 cells produce pro-inflammatory cytokines such as IFN-γ or TNFα that activates macrophages to kill intracellular pathogens. On the other hand, Th2 cells secrete anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 which are important in clearing extracellular pathogens. A deregulation in the balance between Th1 and Th2 cytokines has long been implicated in MS immunopathogenesis (Comabella and Khoury, 2012). Th17 cells represent a distinct, newly discovered lineage of effector T cells. IL-23 produced by macrophages and dendritic cells is critical for expansion of Th17 cells that synthesize the cytokines IL-17A and IL-17F with highly pro-inflammatory properties. Accumulating data suggest that Th17-mediated immune responses are very important in host defense but also in promoting chronic inflammation and autoimmunity (Korn et al., 2009).

In MS, the accumulation of IL-17 producing T cells in the circulation and CNS compartments correlates with disease activity (Becher and Segal, 2011). Studies in EAE have shown that suppression of Th17 cells is associated with a reduction of disease severity, although this is becoming an area of debate. Interestingly, the Th17 to Th1 ratio appears to be a critical determinant of CNS inflammation, and high Th17 to Th1 ratios are associated with T-cell infiltration and inflammation in the brain parenchyma (Comabella and Khoury, 2012).

There is now accumulating evidence that FOXP3 expressing T_{reg} ($CD3^+CD4^+CD25^+$) cells are engaged in the suppression of a variety of physiological and pathological immune responses. Impaired generation of T_{reg} cells or their effector activity may contribute to autoimmune diseases such as MS. This cell population is believed to be important in the induction of remission and prevention of extensive tissue damage during EAE (Korn et al., 2008). Studying human MS patients compared with healthy individuals, the number of circulating T_{reg} cells was shown to be decreased (Khoury et al., 2000) or unchanged (Putheti et al., 2004)

(Haas et al., 2005). A more recent study shows that relative counts of both $CD25^+$ T lymphocytes ($CD3^+CD25^+$) and T_{reg} cells were significantly increased in whole blood of MS patients compared with healthy individuals. Also the relative counts of $CD3^+CD8^+CD25^+$ lymphocytes displayed increased numbers in MS patients compared with those of healthy individuals (Kumar et al., 2006). One possible explanation for this discrepancy could be the definition of T_{reg} cells according to the level of CD25 expression (to include only $CD25^{high}$ or additionally $CD25^{intermediate}$ population). Another explanation might be that different subgroups of MS patients were included in the studies. In vivo depletion of $CD25^+$ T_{reg} cells in a murine EAE model resulted in increased severity, both in terms of mean clinical scores and mortality (Stephens et al., 2005).

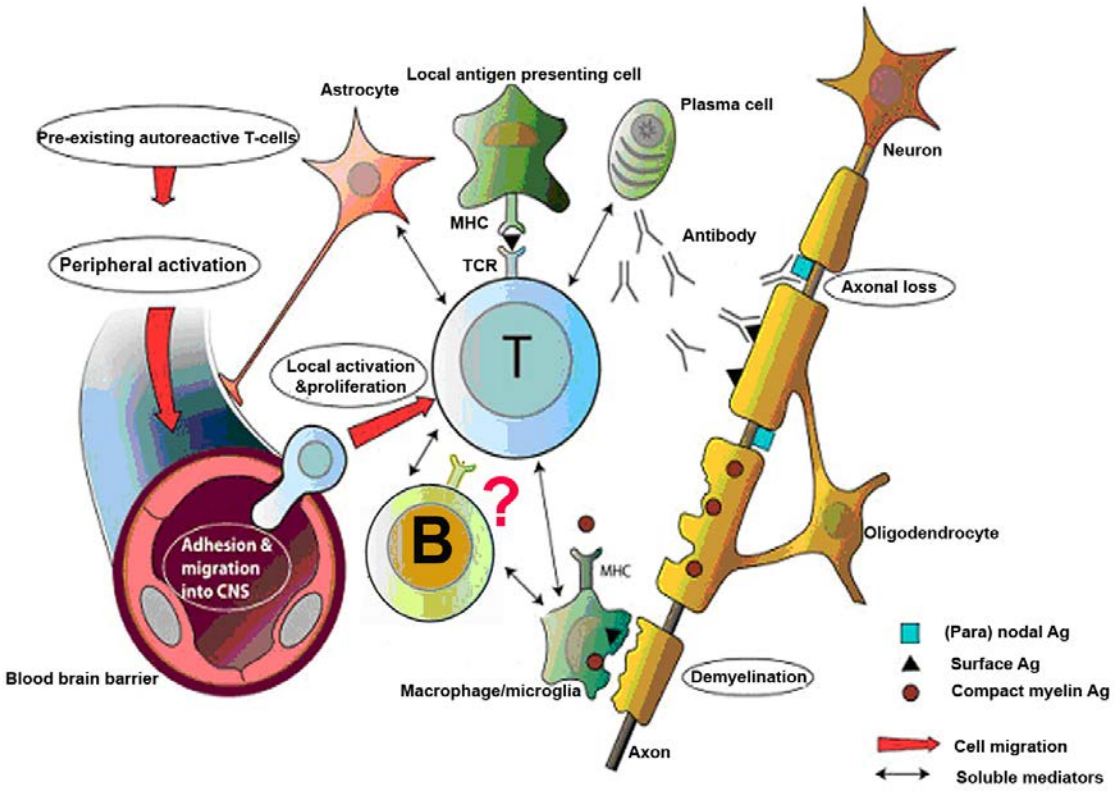


Figure 30. Scheme of MS immunopathogenesis. Pre-existing autoreactive T cells are activated outside the CNS. The activated T cells traverse the blood-brain-barrier and are locally re-activated when they recognize 'their' antigen on the surface of local antigen presenting cells. The activated T cells secrete cytokines that stimulate microglia cells and astrocytes, recruit additional inflammatory cells, and induce antibody production by plasma cells. Anti-myelin antibodies and activated macrophages/microglia cells are thought to cooperate in demyelination (Modified from Hohlfeld et al.; Brain 1997).

There are several lines of evidence suggesting an important role of $CD8^+$ T-cells in MS immunopathogenesis: **(1)** $CD8^+$ T-cells are prominent in the inflammatory infiltrate in human CNS lesions, and in some studies, $CD8^+$ T-cells outnumber $CD4^+$ T-cells; **(2)** infiltrating $CD8^+$ T-cells are clonally expanded and may persist in the CSF for many years; **(3)** $CD8^+$ T-cells may promote CNS vascular permeability; **(4)** axonal damage was correlated with the number of $CD8^+$ T lymphocytes infiltrating the lesion; **(5)** in animal models adoptive transfer of activated myelin-specific $CD8^+$ T-cell clones has been shown to induce EAE, suggesting a role for $CD8^+$ T-cells as effector cells in MS pathogenesis (discussed in (Comabella and Khoury, 2012)).

The first hypothetical event in MS pathogenesis is the initial activation of the autoreactive T-cells in the periphery (reviewed in (Hohlfeld, 1997)). Based on animal experiments in EAE and observations in human MS patients several scenarios have emerged: **(1)** molecular mimicry, during bacterial or viral infection **(2)** cross-reactivity that occur at the level of TCR (in case two types of αβ TCR could form and appear on the cell surface) **(3)** stimulation of viral or bacterial superantigens (Fleischer, 1994) **(4)** high local concentrations of cytokines secreted in the course of unrelated inflammatory reactions. Of note, **(5)** CNS-reactive T-cells may be activated (or reactivated) within the CNS itself. Under normal physiologic conditions lymphocytes are able to traffic into the CNS. Resident microglial cells, both perivascular and parenchymal, have been identified as competent APCs, thus antigen presentation could be accomplished by resident cells or by invading monocytes/macrophages and dendritic cells (Bar-Or, 2008).

3.1.5. Role of the B lymphocytes

The predominant view has held that pathology and disease activity in MS is mediated by T-cells by the peripheral activation of pro-inflammatory $CD4^+$ (Th1, ThIL-17) and $CD8^+$ T cells. However, humoral immunity is also considered to play an important role in MS pathogenesis as indicated by the persistent intrathecal production of oligoclonal immunoglobulins in the CSF from MS patients, a finding that is part of the diagnostic criteria for the disease. B-cells may directly participate in the demyelination process by secreting pathogenic antibodies that target oligodendrocytes with or without the presence of complement (O'Connor et al., 2005).

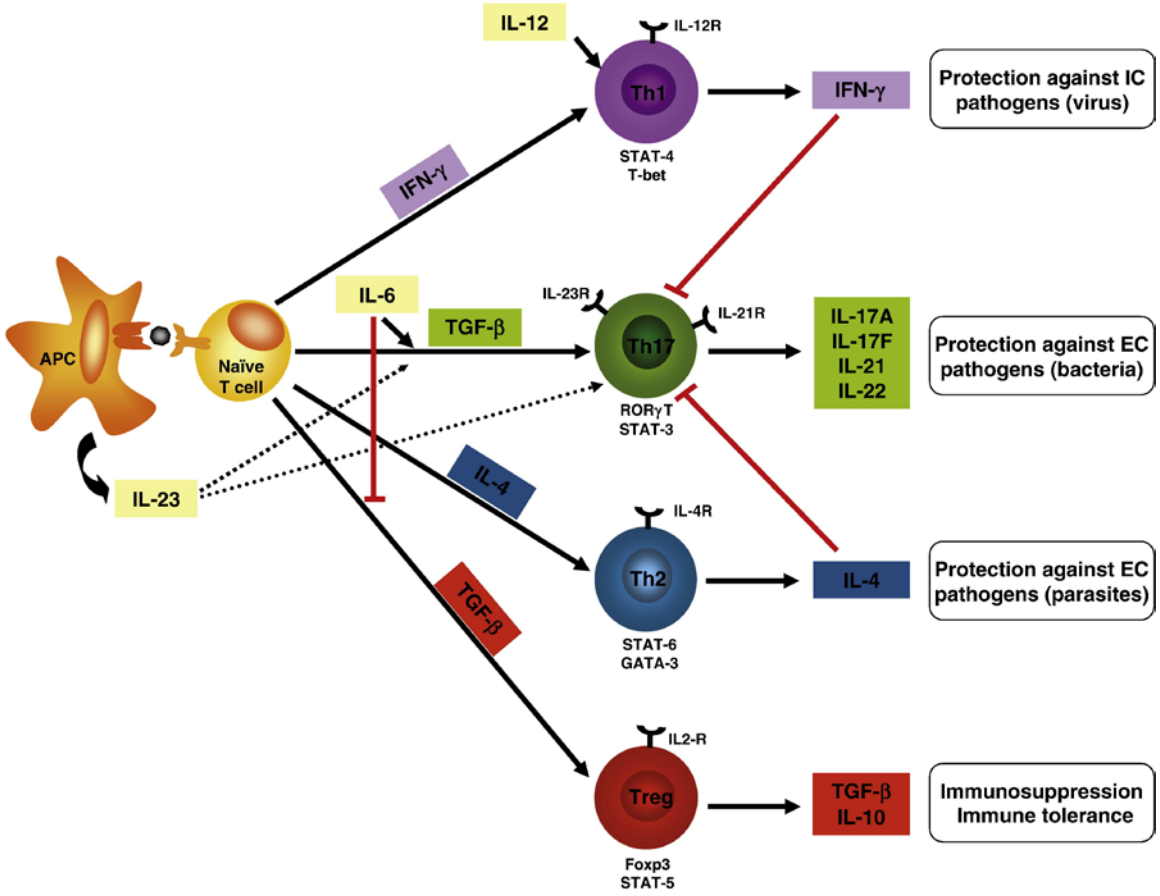


Figure 31. Upon activation, naïve CD4+ T cells differentiate into functionally distinct T cell subsets: Th1, Th2, Th17, and Treg under the influence of specific cytokines. These cell subsets express master transcription factors (shown underneath the cells) that are required for lineage commitment. Antagonistic actions of IL-4 and IFN-γ on Th17 development and of IL-6 on Treg cells are also shown. The fully-differentiated T cell subsets secrete specific cytokines that mediate their effector functions. Th1 cytokines will be important in host defence against intracellular pathogens such as viruses or intracellular bacteria. Th2 cytokines enhance humoral immune responses and will be essential for clearance of extracellular pathogens such as parasites. Th17 cells secrete cytokines with strong pro-inflammatory properties that will be involved in the host defence against extracellular pathogens such as bacteria. Finally, cytokines produced by Treg cells will play positive roles in immunomodulation. EC indicates extracellular; IC, intracellular (Adapted from Comabella et al., 2012).

The presence of tertiary lymphoid organs with germinal centers in the meninges and cerebral perivascular spaces (Prineas, 1979) of some patients with secondary progressive MS suggests that abnormal lymphoid microenvironments can also form and can be maintained locally in an immune privileged site like the CNS and may contribute to the pathogenic process (Magliozzi et al., 2007). These lymphoid structures resemble ectopic lymphoid follicles, with FDC networks, CXCL13-expressing stromal cells, proliferating B-cells and plasma cells. These intrameningeal follicles were observed in patients with secondary progressive MS, but not in

relapsing remitting and primary progressive MS (Serafini et al., 2004). It has been recently shown that the presence of these B-cell follicle-like structures correlates with an earlier onset and more severe disease (Franciotta et al., 2008). The development of such ectopic follicles was also shown in SJL mice with relapsing EAE, but could not be detected in C57BL/6 or DBA-1 mice after immunization (Magliozzi et al., 2004).

Recently, clinical trials of a B-cell ablative therapy with rituximab, an anti-CD20 monoclonal antibody (which efficiently depletes naïve and memory B-cells), demonstrated a significant reduction in new focal inflammatory brain lesions and clinical relapses in patients with RRMS (Hauser et al., 2008). Importantly anti-CD20 treatment is independent of secreted antibodies since Rituximab does not directly target plasma cells, which implicates an antibody-independent role of B-cells in the disease activity. The three primary antibody secretion-independent ways that B-cells potentially impact T-cell activation or regulation are by **(1)** releasing cytokines and chemokines, thus facilitating a local microenvironment favourable to evolving cellular autoimmune responses, **(2)** providing co-stimulatory signals through direct B-cell – T-cell interaction (CD40, OX40 or CD 80/86 mediated interactions), or **(3)** serving as antigen presenting cells (Ireland and Monson, 2011) (Pollinger et al., 2009) (**Figure 32**).

B-cells are capable of producing a wide variety of pro-inflammatory and regulatory cytokines and growth factors that have long been appreciated for their potential to influence B- and T-cells. Among other important cytokines, B-cells can produce TNFα and LTα, which, in addition to IL23, are important factors for potentiating Th17 responses (Ireland et al., 2012).

A recent study reports that B-cells of MS patients exhibit abnormal pro-inflammatory cytokine responses (including exaggerated production of LT and TNFα), when activated in the context of the Th1 cytokine interferon IFNγ or in presence of TLR9 ligand (CpG DNA) (Bar-Or et al., 2010). Furthermore, the depletion of B-cells *ex vivo* and *in vivo* (in two clinical trials of rituximab in RRMS) results in significantly reduced pro-inflammatory responses of both CD4 and CD8 T-cells. Importantly they confirmed that secreted factors produced by activated B-cells from RRMS patients before treatment can reconstitute the diminished T-cell responses observed following *in vivo* B-cell depletion in the same patients. This finding implies that the effect is mediated at least partially by B-cell derived LT and TNFα.

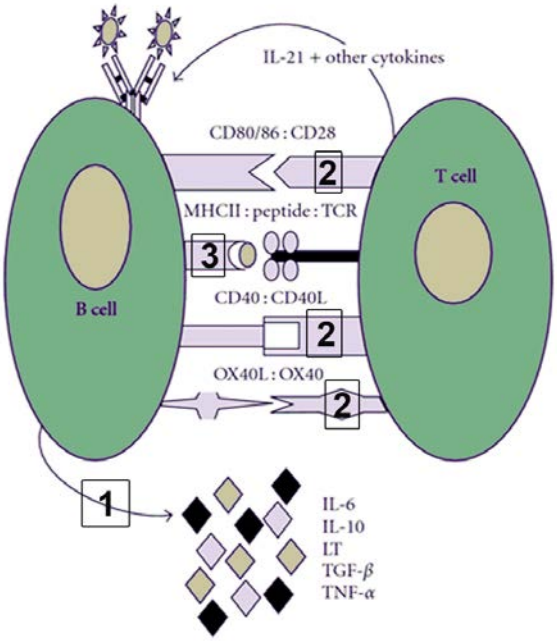


Figure 32. Antibody independent roles of B-cells in T-cell activation. (1) releasing cytokines and chemokines, thus facilitating a local microenvironment favourable to evolving cellular autoimmune responses, **(2)** providing co-stimulatory signals through direct B-cell – T-cell interaction (CD40, OX40 or CD 80/86 mediated interactions), or **(3)** serving as antigen presenting cells. Adapted from (Ireland and Monson, 2011).

3.1.6. Effects of LT in various EAE models/ in demyelination

Increased levels of LT has been demonstrated during the course of human MS on mRNA and protein level **(1)** in the cerebrospinal fluid (CSF) of MS patients (Matusevicius et al., 1996) **(2)** in the demyelinated plaques (Raine et al., 1998) **(3)** and in the meninges and white matter lesions (Selmaj et al., 1991a) (Lassmann et al, unpublished observations). In vitro studies indicate that LTα has a direct cytotoxic role on oligodendrocytes (Selmaj et al., 1991b).

The presence of LT has been reported in association of $CD3^+$ lymphocytes and microglial cells in acute and chronic active MS brain lesions (Selmaj et al., 1991a), and is therefore, suggested to be important in the development of demyelination. In the cuprizone induced model of demyelination it was shown that presence of LTα – produced by activated astrocytes – exacerbated demyelination and that the lack of LTα did not alter the course of remyelination (in contrast to the lack of TNFa) or the proliferation of oligodendrocyte progenitors during the remyelination phase of the cuprizone model (Plant et al., 2005). Further, also enhanced expression of LTβR mostly on microglia during the demyelination phase was demonstrated (Plant et al., 2007). This result suggests that LTαβ–LTβR signalling between astrocytes and microglia is a primary mediator in the inflammatory demyelinating process that occurs during cuprizone treatment.

EAE has been induced in several knockout ($LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $TNF/LT\alpha^{-/-}$) mouse models to examine the contribution of LTβR pathway to multiple sclerosis, often with conflicting results. A complicating factor in several studies is that disruption of these genes causes developmental defects, structural and functional immune deficiencies, such as abnormal lymph nodes, altered splenic architecture, and abnormal immune function (Frei et al., 1997) (Suen et al., 1997) . In a more recent study immunodeficiency was corrected in $LT\alpha^{-/-}$ mice by reconstitution with bone marrow cells (Sean Riminton et al., 1998). However, this "rescue" could be influenced by the side effects of irradiation on the integrity of the blood brain barrier (BBB). To dissect the role of LT in a system with a full complement of lymph nodes and intact lymphoid microenvironment, LTβR pathway was blocked with the fusion protein LTβR-Ig in the context of three different EAE models (Gommerman et al., 2003). Administration of LTβR-Ig prior to immunization with myelin peptides inhibited disease development in a rat monophasic EAE model and prevented late-stage disease relapses in a mouse model of relapsing EAE (**Figure 33**).

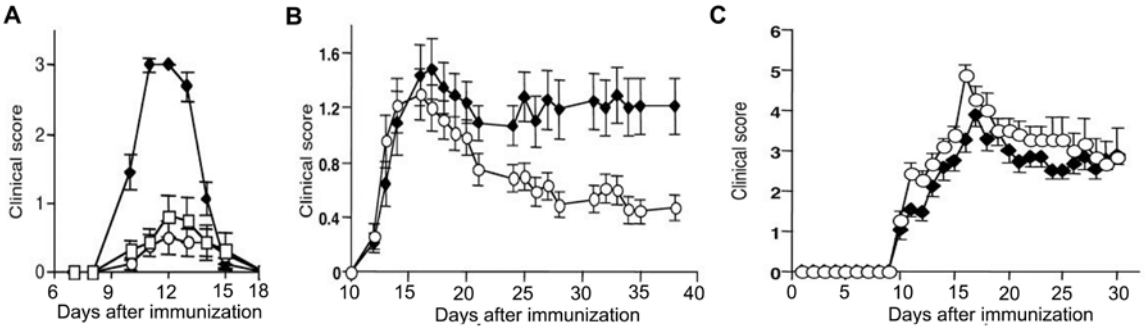


Figure 33. Effect of LTβR pathway inhibition in different models of EAE. (A) Lewis rats were treated with either control human IgG (diamonds), or LTβR-Ig (circles) 1 day prior to immunization with MBP-peptide in CFA. **(B)** Relapses - but not acute disease - are prevented by LTβR-Ig treatment in a chronic murine EAE model. SJL mice were treated with control huIgG (diamonds) or LTβR-Ig (circles) 1 day prior to immunization with $PLP_{139–151}$ in CFA. **(C)** LTβR-Ig treatment does not prevent EAE in a PTx-dependent model. C57BL/6 mice were treated with control huIgG (diamonds) or LTβR-Ig (circles) 1 day prior to immunization with $MOG_{35–55}$ in CFA followed by injection of PTx on days 0 and 2 (Adapted from Gommerman et al., 2003).

The authors also proved that disease prevention by LTβR-Ig was due to specific blockade of $LT\alpha_1\beta_2$ binding, rather than LIGHT binding to the LTβR. In addition treatment with LTβR-Ig was shown to inhibit T-cell infiltration, formation of organized ectopic B-cell follicles and synthesis of inflammatory (CXCL10) and lymphoid (CXCL13) chemokines in the inflamed CNS during the course of EAE in SJL mice (Columba-Cabezas et al., 2006).

3.2. Scientific aims

Although the above described data about the role of LT and chemokines in the CNS indicate an important role of LT in autoimmune diseases of the CNS, the identity of cells expressing LT as well as the cells receiving and integrating LT remain elusive.

The goal of this project was to **(1)** generate a mouse model *(GFAP-Lta,Ltb)* to study the role of astrocyte derived LT in various diseases of the CNS **(2)** to investigate the contribution of LT induced cytokine production and tissue destruction in CNS autoimmunity as well as **(3)** to examine the role of LTβR on specific brain cells in the various models of autoimmune disease by using $LT\beta R^{loxP/loxP}$ mice (Cuprizone, EAE). Furthermore *Tg(GFAP-Lta,Ltb)* mice were crossed with mice bearing myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic T-cells (GFAP/TCR) to investigate if double transgenic mice will develop spontaneous inflammatory demyelinating disease.

In future studies, *Tg(GFAP-Lta,Ltb)* mice could be used to induce primary CNS lymphoma in mice (PCNSL). We hypothesize, that the inflammatory environment in the CNS as the consequence of LT over-expression would provide an appropriate milieu for adoptively transferred B-lymphoma cells derived from Emu c-myc mice.

3.3. Results

3.3.1. Expression of receptors and ligands of the core TNF superfamily in the CNS

Several studies attributed an important role to LT in inflammatory CNS diseases. However, due to the lack of specific antibodies directed against lymphotoxin and its cognate receptor, LTβR, the cell specific expression patterns of these molecules in the CNS remains elusive. Therefore, I have tested the expression of the receptors of the core TNF superfamily, TNFR1, TNFR2 and LTβR on purified neural cell populations (astrocytes, neurons, oligodendrocytes and microglia) from C57BL/6 mice by qPCR analysis (**Figure 34**). Purity of the cells was confirmed by RT-PCR analysis of neural markers compared to cortical expression of a wild type mouse (Astrocytes: GFAP; Oligodendrocytes: MBP; Neurons: β-tubulin III; Microglia: CD11b) (data not shown). The receptors, *Ltβr*, *Tnfr1* and *Tnfr2* are mainly expressed on oligodendrocytes and microglial cells, while *Ltα* and *Light* shows predominantly neuronal and

microglial expression pattern. In contrast *Tnfα* appears to be highly expressed on oligodendrocytes.

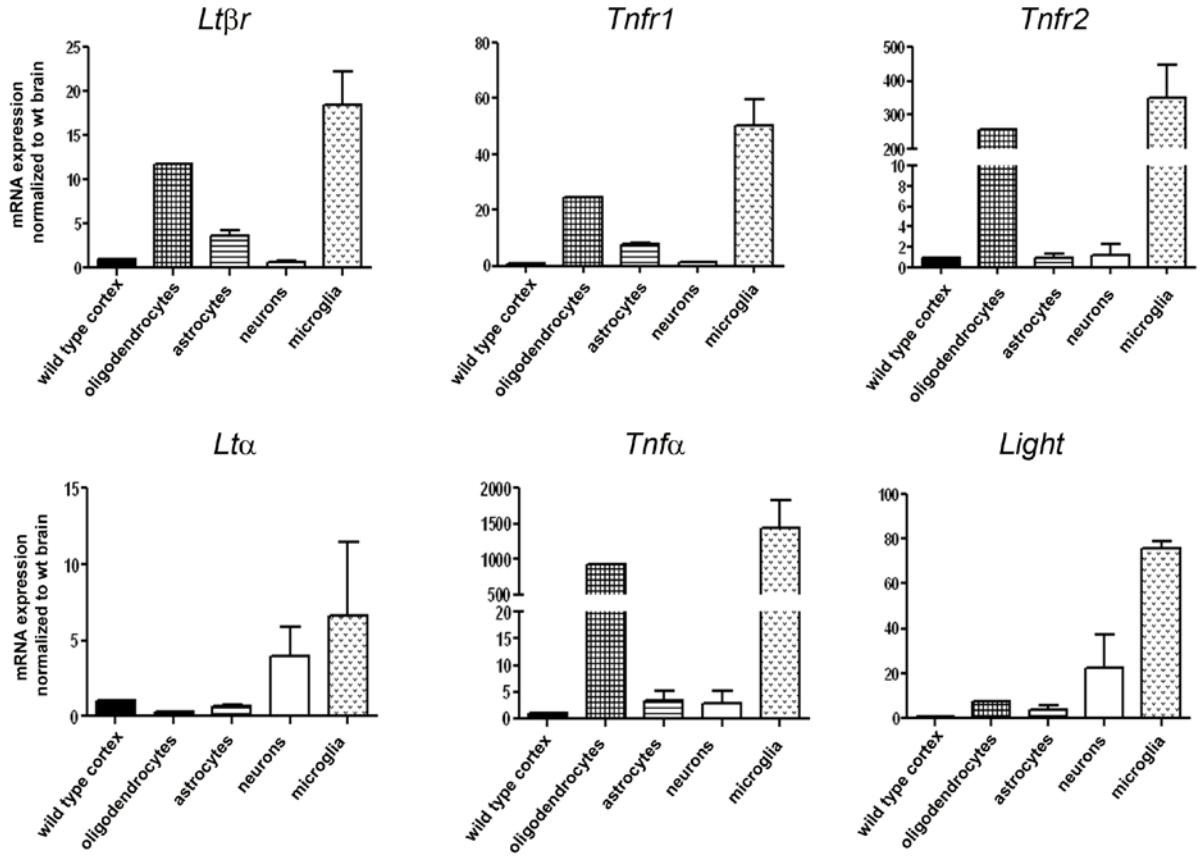


Figure 34. Distribution of expression of TNF family ligands and receptors on different neural cell types. Relative mRNA transcript expression measured by RT-PCR analysis *of LTβR, TNFR1, TNFR2, LTα, TNFα* and *Light* on oligodendrocytes, astrocytes, neurons and microglia isolated from C57BL/6 mice. mRNA expression in the different cells populations was compared to mRNA isolated from the cortex of C57BL/6 mice.

3.3.2. Generation of transgenic mice

Earlier findings indicate that LT expression is upregulated during the course of human MS (Raine et al., 1998) (Selmaj et al., 1991a), furthermore studies by Plant et al., claim that the absence of lymphotoxin turns mice highly resistant to Cuprizone induced demyelination. In order to elucidate the direct role of LT in demyelination and CNS inflammation I generated double transgenic mice on C57BL/6N background expressing both LTα and LTβ under the control of the human glial fibrillary acid protein promoter *(Tg(GFAP-Lta,Ltb))* **(Figure 35)** (Brenner et al., 1994) that drives the expression specifically on astrocytes. Three double transgenic, *Tg(GFAP-Lta,Ltb)* founder mice were identified expressing the respective

transgenes and subsequently bred to generate three independent transgenic lines *((GFAP-Lta,Ltb)#8*; *(GFAP-Lta,Ltb)#23; (GFAP-Lta,Ltb)#26))*. The litter size and the average weight of mice were normal, and mice displayed normal behaviour and growth. Since no gender dependent qualitative differences were found, both male and female mice were used in the further described studies.

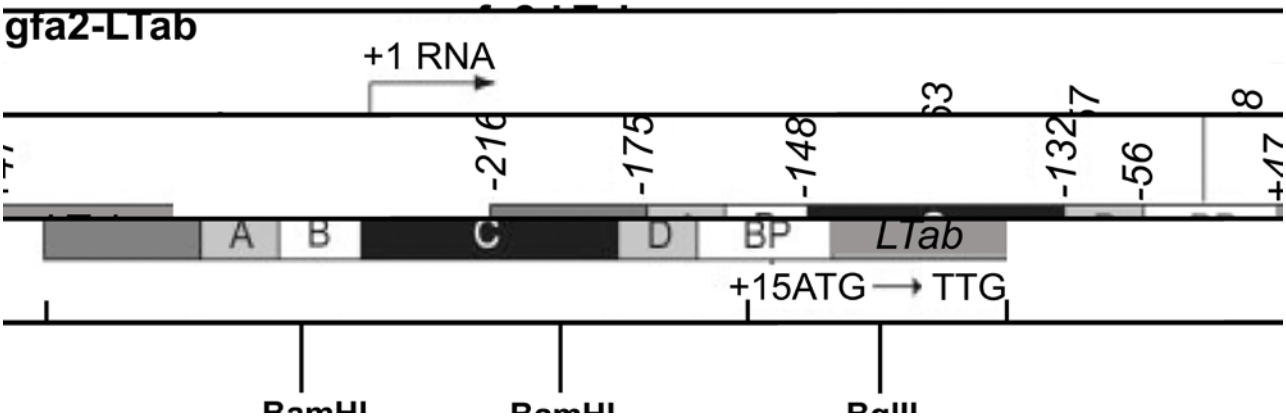


Figure 35. GFAP –Lta,Ltb transgene structure. The gfa2 promoter contains the human GFAP fragment spanning bp -2163 to +47 relative to the transcriptional start site (Brenner et al., 1994).The promoter has the natural initiating ATG at bp +15 converted to TTG so that protein synthesis initiates within lymphotoxin**.** Adapted from (Lee Y. et al., Glia 2008). BamHI restriction sites, used for the cloning strategy are indicated.

The level of lymphotoxin expression was different between the three transgenic lines (**Figure 36A**), thus we classified *Tg(GFAP-Lta,Ltb)#8* as the low expresser, *Tg(GFAP-Lta,Ltb)#23* as the intermediate expresser and *Tg(GFAP-Lta,Ltb)#26* as the high expresser line. In each transgenic line cortex, cerebellum, brain stem and spinal cord (n=2-3) were analysed for endogenous LT mRNA expression. Among the different brain regions analysed, the brain stem and the spinal cord shows the highest LT expression in the intermediate and high expresser transgenic lines compared to wild type littermates.

Thorough RT-PCR analysis revealed that in *Tg(GFAP-Lta,Ltb)* mice transgene expression is restricted to the CNS, since no LT expression could be detected in other organs such as spleen, kidney (**Figure 36B**). Slightly elevated LTα expression was observed in the liver, which did not cause any pathological changes up to 6 months of age (data not shown). This finding is in accordance to several studies, showing that GFAP-driven transgenes may also be active outside of the CNS. Endogenous GFAP expression has been reported for several non-CNS tissues, albeit at levels generally much lower than in astrocytes. These include non-

myelinating Schwann cells, fibroblasts, liver perisinusoidal stellate cells, respiratory tract chondrocytes and lymphocytes (Su et al., 2004).

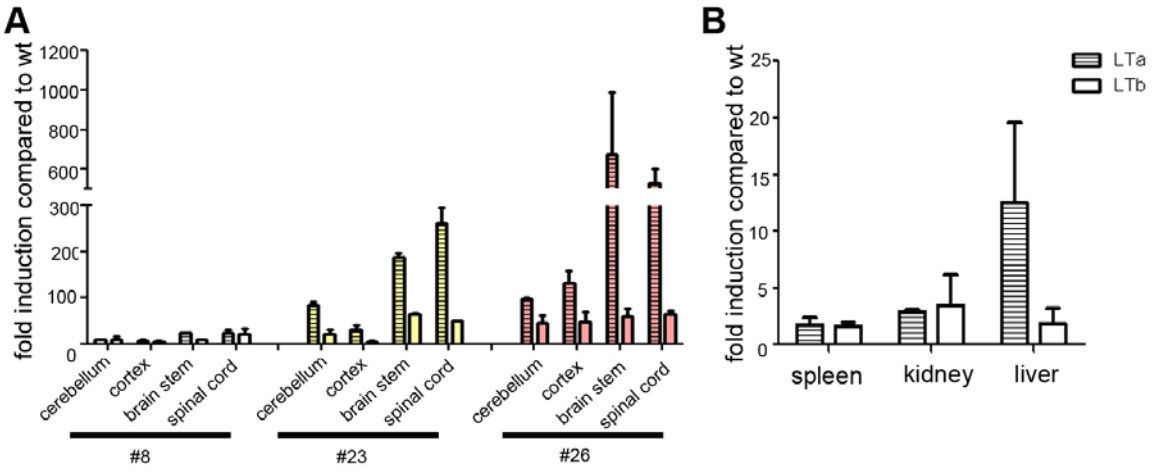


Figure 36. Lymphotoxin expression in three independent double transgenic lines. (A) Endogenous LT mRNA expression was analyzed in the cerebellum, cortex, brain stem and spinal cord samples from *(GFAP-Lta,Ltb)#8*; *(GFAP-Lta,Ltb)#23 and (GFAP-Lta,Ltb)#26 transgenic lines.* **(B)** LT expression is highly specific to the CNS, no or very little elevated LT mRNA expression was found outside of the CNS in *(GFAP-Lta,Ltb)#23* mice (spleen, kidney), however a slight upregulation of LTa in the liver was observed, likely due to the activity of the GFAP promoter on hepatic stellate cells (Ito cells).

3.3.3. Astrocyte specific LT over-expression causes early onset of reactive gliosis

I first investigated by immunohistochemistry whether LT overexpression on astrocytes will cause pathological changes in the CNS. All three double transgenic lines develop astrogliosis already at the age of two months visualized by GFAP staining. The severity of gliosis correlates with the relative amount of transgene expression (**Figure 37**).

Based on the similar patterns in the observed gliosis in all three transgenic lines I could exclude that the phenotype of *Tg(GFAP-Lta,Ltb)* mice is a transgenic artefact. Due to the higher transgene expression levels in *Tg(GFAP-Lta,Ltb)*#26 mice I focused for the further analyses on this transgenic line. However, the observed phenotype and functional experiments were also corroborated in the intermediate expresser line. Besides the astrogliosis further histopathological analysis from 3 months old *Tg(GFAP-Lta,Ltb)*#26 mice and negative littermates in the nucleus caudatus revealed enhanced microglia activation (Iba-1) however no phagocytic activity (Mac3). Pronounced astrogliosis can be observed (GFAP), in addition the astrocyte polarity shows a different distribution compared to wild type mice; Aquaporin 4 is reduced in glia limitans but enhanced in astroglial cell bodies in transgenic mice. AQP4 is a

water-selective membrane transport protein expressed in astrocytes, strictly distributed in a polarized fashion on the astrocyte endfeets.

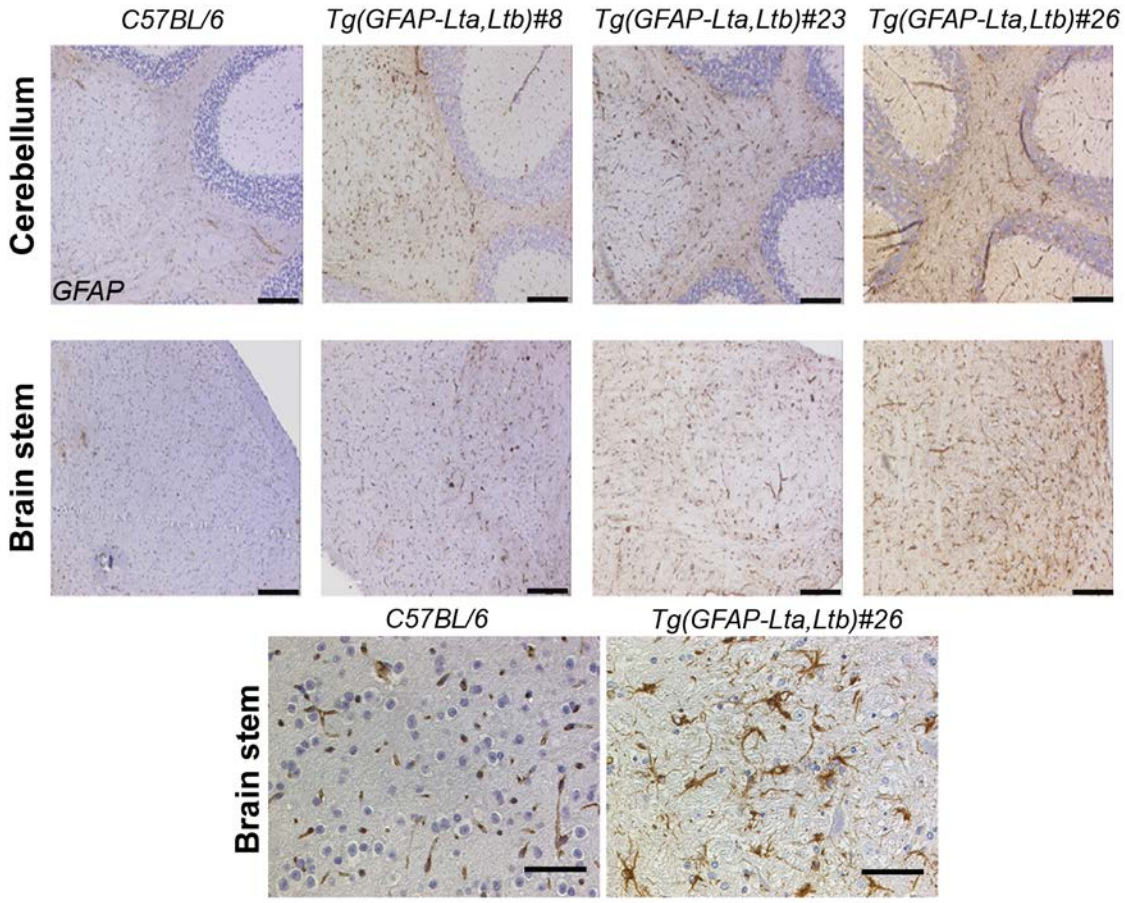


Figure 37. Reactive astrogliosis in *Tg(GFAP-Lta,Ltb)* mice. GFAP immunoreactivity reveals astrogliosis in all three double transgenic lines at the age of two months in the cerebellum and brain stem. The prominence of astroglial reaction in various *Tg* lines correlates with the expressed amount of LTα and LTβ (scale bar: 200 μm). Lower panel displays high magnification pictures about GFAP staining in the brain stem of wild type and *Tg*(GFAP-Lta,Ltb) mice (scale bar: 50 μm).

Under normal conditions AQP4 is involved in brain water balance, neuroexcitation and astrocyte migration (Li et al., 2011). However, during EAE, within the inflammatory cuff, AQP4 redistributes from the endfeet membranes to the parenchymal membranes of the astrocyte body. The degree of both alterations was shown to correlate with the amount of CNS inflammation and clinical severity of EAE (Wolburg-Buchholz et al., 2009).

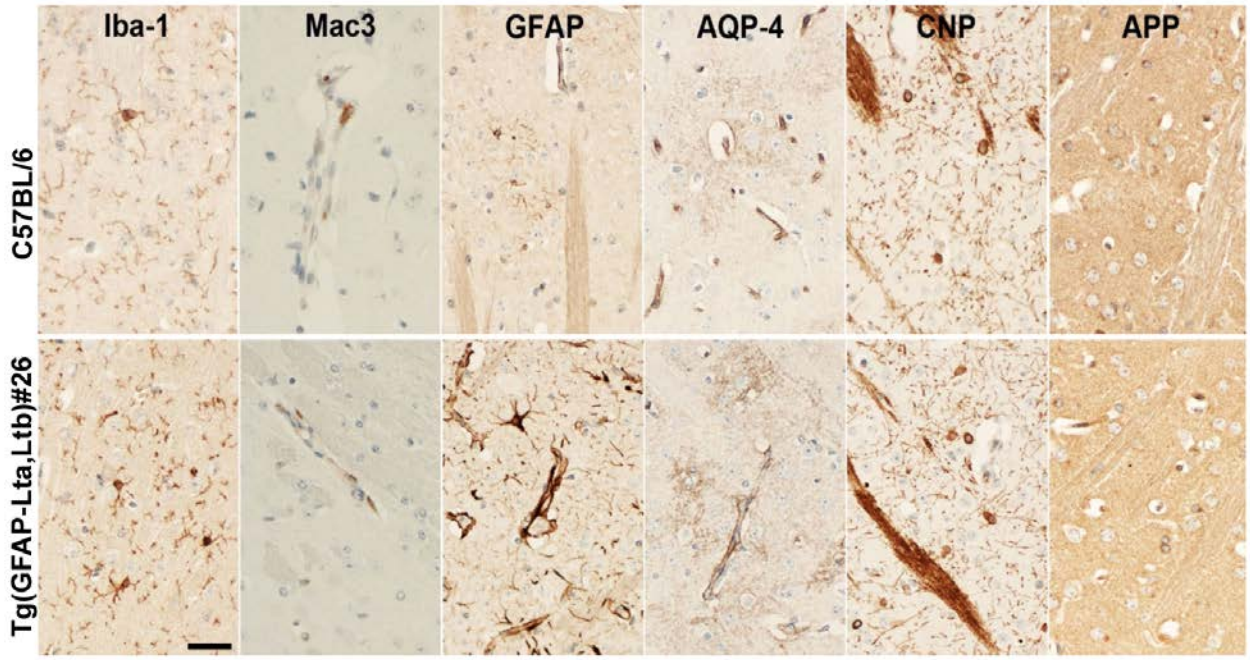


Figure 38. Detailed histological analysis of *Tg*(GFAP-Lta,Ltb)#26 mice. Special stainings from 3 months old C57BL6 and *Tg*(GFAP-Lta,Ltb) mice in the nucleus caudatus. Transgenic mice show enhanced microglia activation (Iba-1) and astrogliosis (GFAP). Macrophages are visualized by Mac-3 staining, astrocyte polarity by Aquaporin 4. Oligodendrocytes and myelin sheet remains unchanged (CNPase) and no axonal damage can be found (APP) in the *Tg*(GFAP-Lta,Ltb) mice (scale bar: 50 μm).

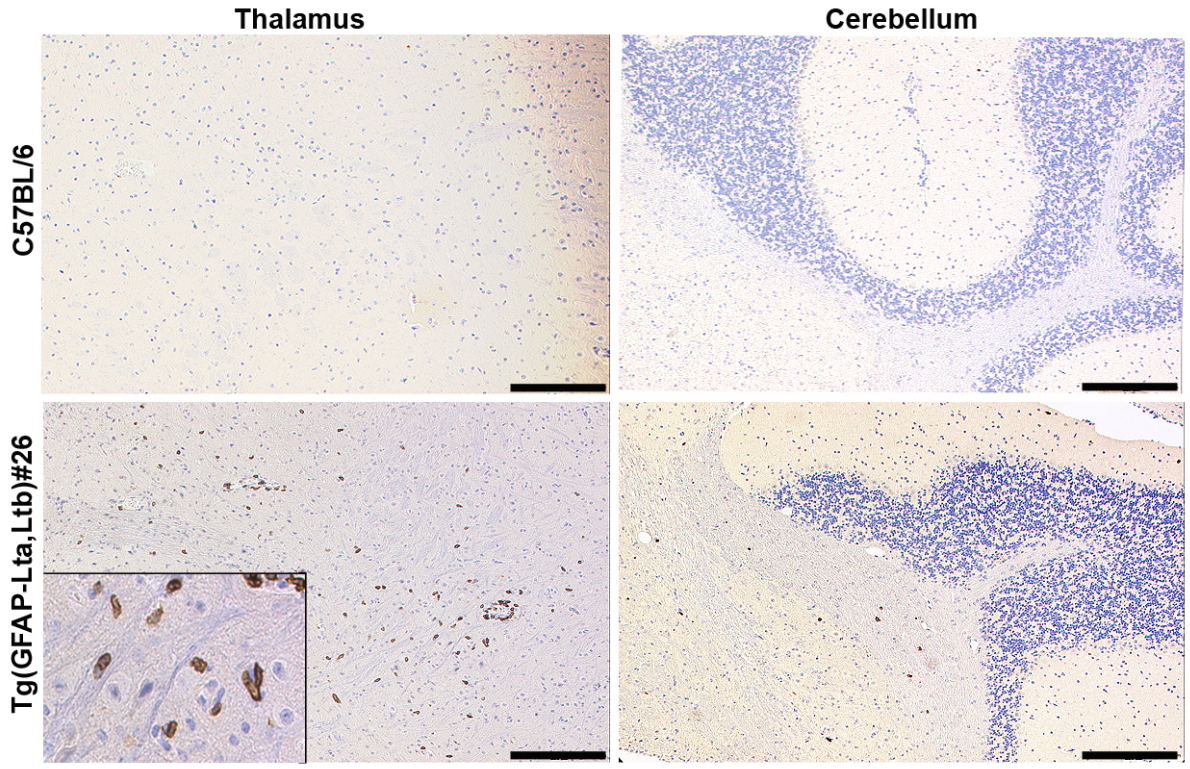


Figure 39. T-cell infiltrates in the thalamus and cerebellum of *Tg*(GFAP-Lta,Ltb)mice. **N**umerous infiltrating $CD3^+$ cells in *Tg* mice compared to negative littermates (scale bar: 200 μm) .

In *Tg*(GFAP-Lta,Ltb) mice oligodendrocytes and myelin sheet remains unchanged (CNPase) and no dystrophic axons can be found (APP expression) (**Figure 38**). Furthermore infiltration of $CD3^+$ lymphocytes in the CNS parenchyma can also be found starting at three month of age (**Figure 39**). Infiltrating B-cells (B220) were not observed at any time point in any of the analysed CNS regions (data not shown).
The most prominent pathological changes can be seen in the small cerebellar nuclei, thalamus and basal ganglia whereas the cortex and white matter show the least severe phenotype.

3.3.4. The blood brain barrier is intact in three months old transgenic mice

The observed microglial activation and astrogliosis suggest a starting neuroinflammation in the CNS of *Tg(GFAP-Lta,Ltb)* mice. Inflammation in the CNS can be coupled with an increased permeability of the blood brain barrier (BBB). The BBB is a selective barrier that maintains homeostasis of the brain. Recent work has uncovered a critical role for pericytes in the integration of endothelial and astrocyte functions at the neurovascular unit, and in the regulation of the BBB (Armulik et al., 2010). The BBB acts as a 'physical barrier' because it is formed by the arrangement of tight junctions between adjacent endothelial cells that line cerebral microvessels. Small gaseous molecules such as O_2 and CO_2 can diffuse freely through the lipid membranes, and this is also a route of entry for small lipophilic agents, including drugs such as barbiturates and ethanol (Abbott et al., 2006). The healthy CNS vasculature is impermeable to the passive transport of cells, large hydrophilic molecules such as peptides and proteins (like serum albumin), and bioactive compounds present in the blood (Armulik et al., 2011). Therefore the integrity of the BBB was tested in *Tg(GFAP-Lta,Ltb)* mice. First, brain sections from 3 months old wild type and transgenic mice were stained to visualize endogenous serum albumin extravasation in brain parenchyma (**Figure 40A**). No immunoreactivity against albumin was found in the brain of transgenic mice suggesting the presence of an intact BBB. To provide further confirmation that BBB permeability is not altered we injected Evans blue (a dye that has a very high affinity to serum albumin) intravenously into wild type and *Tg* mice (**Figure 40B**). The experiment was done in collaboration with Prof. Marco Prinz in Freiburg. The lack of blue colour in wild type and *Tg(GFAP-Lta,Ltb)* brains and spinal cords suggests no gross alteration in BBB permeability.

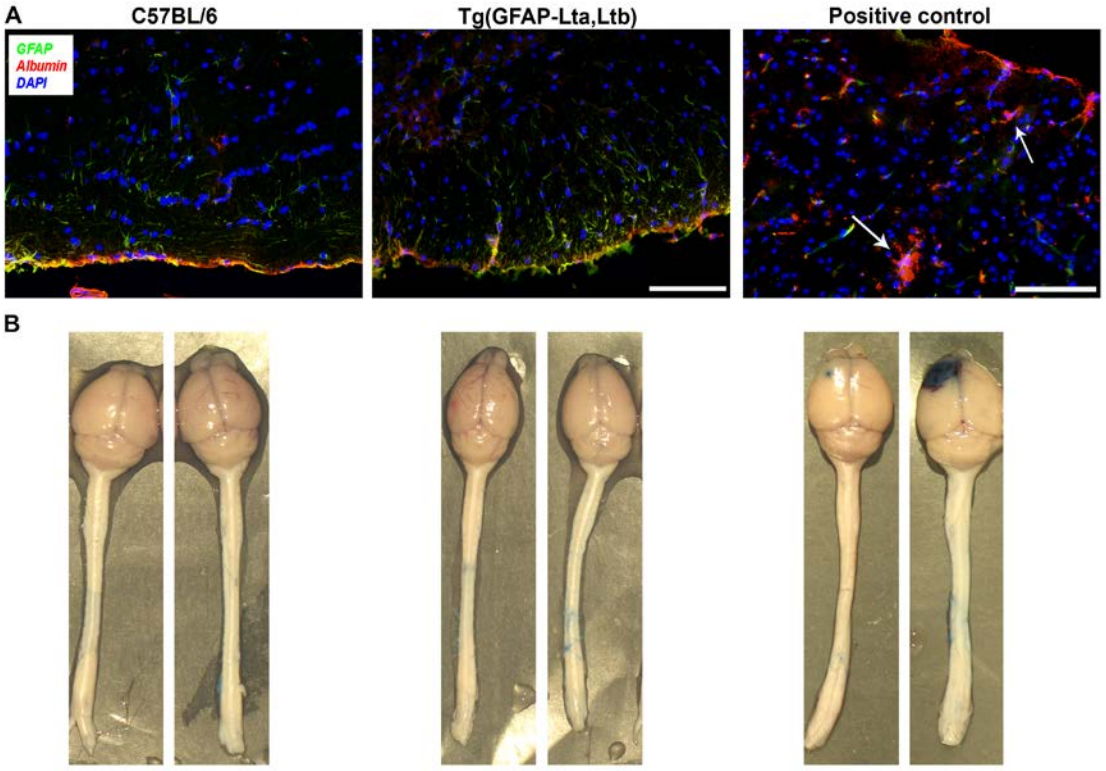


Figure 40. Integrity of the blood-brain barrier. (A) Immunofluorescent staining of extravasated serum albumin in brain sections of wild type and transgenic mice. *C57BL/6* mice 14 days post irradiation served as a positive control. Positive albumin staining (white arrows) indicates increased blood-brain barrier permeability in the irradiated mice, but not in *Tg(GFAP-Lta,Ltb)* mice. **(B)** Whole brains and spinal cords photographed after Evans blue injection. No BBB damage can be seen in wild type or transgenic mice. The blue spots on the spinal cord of transgenic mice indicate the presence of meningeal vessels. Mice that suffered mechanic brain injuries were used as positive control.

3.3.5. Lymphotoxin over expression in the CNS leads to a local inflammatory milieu

LT was shown to play an important role in CNS diseases by being directly cytotoxic to oligodendrocytes (Selmaj et al., 1991b). However LT can regulate the expression of inflammatory cytokines, homeostatic (lymphoid) chemokines and adhesion molecules, which are likely to contribute to the development of an inflammatory environment. To test if increased lymphotoxin expression on astrocytes can lead to the generation of an inflammatory milieu within the CNS, we investigated chemokine and cytokine expression on mRNA and protein level in different brain regions of the *Tg(GFAP-Lta,Ltb)* mice (**Figure 41**). Evidence is accumulating that lymphoid chemokines (CCL19, CCL21 and CXCL13) play pleiotropic roles when expressed in the CNS. During homeostasis, CCL19 and CCL21 may facilitate immune surveillance of the subarachnoid space via recruitment of central memory T cells.

Conversely, ectopic expression of these chemokines by CNS resident cells was associated with triggering microglial activation and/or the recruitment and organization of infiltrating leukocytes. These chemokines are induced *de novo* (CXCL13) or above baseline levels (CCL19 and CCL21) in response to certain CNS infections and neoplasms, as well as in the setting of autoimmune inflammation. In addition to regulating leukocyte entry across the BBB, they have been associated with the development of ectopic lymphoid structures in the meninges during chronic EAE and secondary progressive MS (Lalor and Segal, 2010). In three months old *Tg(GFAP-Lta,Ltb)* I found significant induction of *Ccl19* and *Cxcl13* mRNA, however *Ccl21* remained unchanged (**Figure 41A** and data not shown). The inflammatory chemokines CXCL9 and CXCL10 has been also demonstrated to be produced in many diverse pathological conditions of the CNS, such as bacterial and viral meningitis, multiple sclerosis, optic neuritis and Alzheimer's disease (Muller et al., 2010). More recent attention has focused on the function of these chemokines in the CNS inflammation. Here I demonstrate that in *Tg(GFAP-Lta,Ltb)* mice inflammatory chemokines, like *Cxcl1, Cxcl9* and *Cxcl10* are induced upon astrocytic LT overexpression (**Figure 41A**). According to the widely held view, CNS immune surveillance is principally mediated by interactions between $\alpha_4\beta_1$-integrin bearing lymphocytes and VCAM-expressing endothelium (Engelhardt et al., 1998). Therefore I have examined the expression of the adhesion molecule *Vcam* in *Tg(GFAP-Lta,Ltb)* mice. Indeed three months old transgenic mice showed an up-regulated *Vcam* mRNA expression, especially in the spinal cord. Furthermore, the highest up-regulation of mRNA transcripts and protein levels (**Figure 41B**) of the described chemokines are found in the cerebellum and spinal cord, which corresponds to the distribution of high LT expression. The levels of CXCL10 and CXCL13 up-regulation were corroborated on protein level as well, supporting the finding that these chemokines are elevated in a LT dependent manner.

So far no indications of spontaneous autoimmunity or demyelination have been observed.

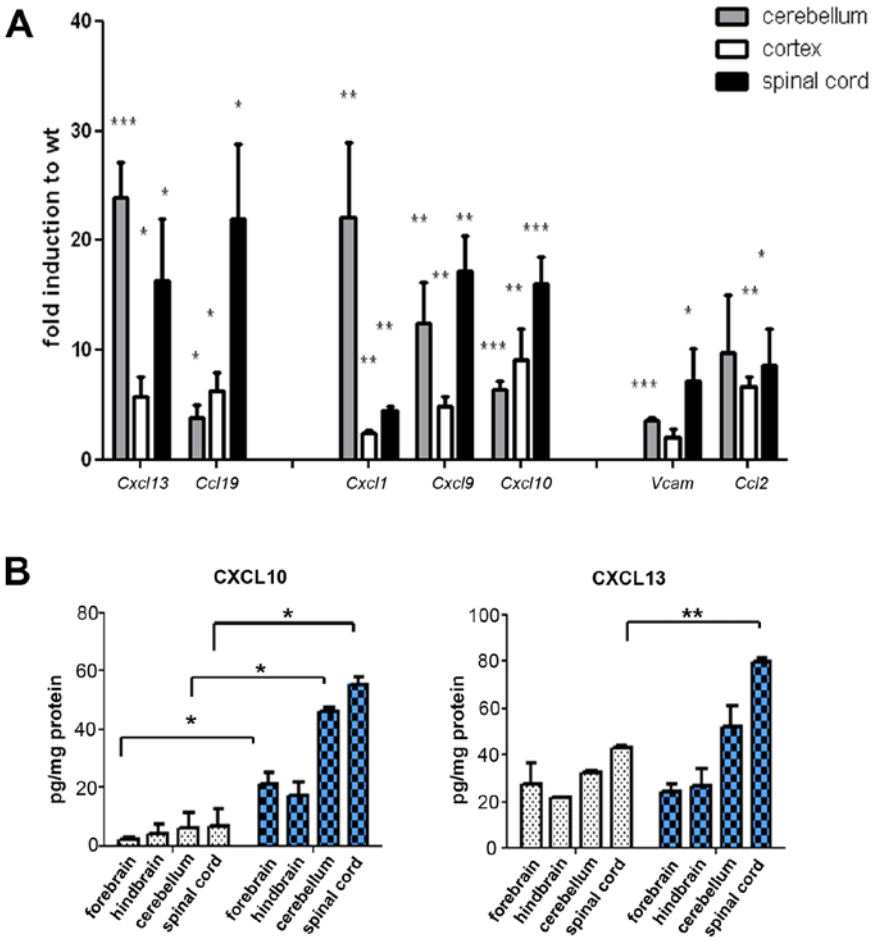
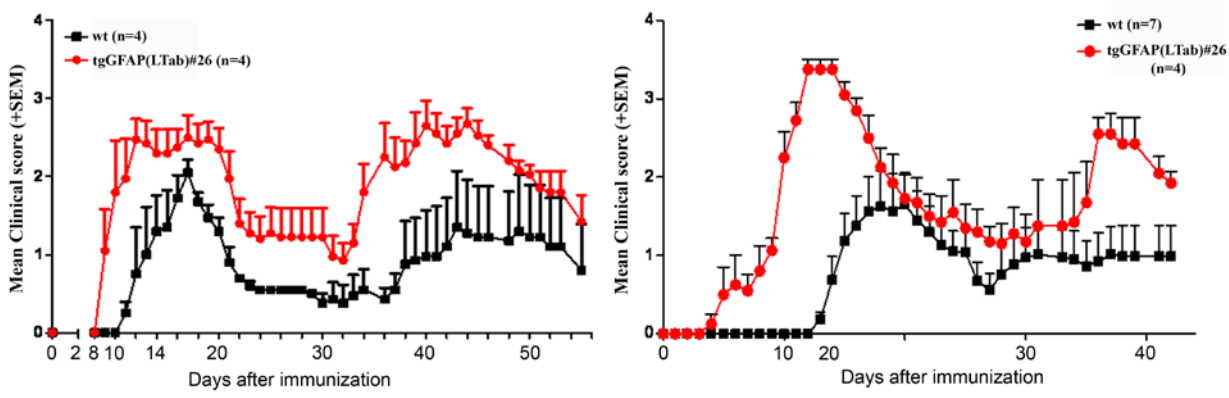


Figure 41. Cytokine and chemokine expression in certain regions of *Tg(*GFAP-Lta,Ltb) mice. (A) mRNA transcript expression of homeostatic chemokines (Cxcl13, Ccl19), inflammatory chemokines (Cxcl1, Cxcl9, Cxcl10, Ccl2) and adhesion molecule (Vcam) in the cortex, cerebellum and spinal cord of 3 months old high LTαβ expresser transgenic line, *Tg(*GFAP-Lta,Ltb)#26. **(B)** Cytokine and chemokine expression on protein level, measured in homogenized brain tissue from the forebrain, hindbrain, cerebellum and spinal cord. *: $P>0.05$; **: $P<0.01$, ***: $P<0.001$

3.3.6. Exacerbated EAE in *Tg*(GFAP-Lta,Ltb) mice

So far all experiments aiming to dissect the role of lymphotoxin in demyelinating diseases utilized either genetic ablation of ligands ($LT\alpha^{-/-}$, $LT\beta^{-/-}$) and receptors ($LT\beta R^{-/-}$) of the LTβR signalling cascade, or applied pharmacological blocking of the LTβR with the LTβR-Ig fusion protein. Although in the beginning the results were slightly controversial due to the developmental abnormalities that LT knockout mice harbor, the utilization of LTβR led to the conclusion in both the cuprizone induced model of demyelination (Plant et al., 2007) and in different EAE models (Gommerman and Browning, 2003), that blocking LTβR-signalling

profoundly inhibits or delays demyelinating pathologies and reduces relapses. In order to assess the direct role of focal lymphotoxin expression, and the LT induced chemokine production in the CNS in autoimmunity, we performed active EAE experiment with *Tg(GFAP-Lta,Ltb)#26* mice. The experiments were done in collaboration with Prof. Marco Prinz in Freiburg. Transgenic mice and wild type littermates were immunized with $MOG_{35–55}$ in CFA. Two independent experiments were performed with 4-7 animals per group (**Figure 42**). In both experiments disease onset after immunization was significantly earlier in transgenic mice (6.5 and 9.5 days versus 13 and 14.5 days) compared to negative littermates, and the incidence was 100%. Besides the maximum clinical score as well as the accumulated clinical score were significantly higher in *Tg(GFAP-Lta,Ltb)#26* mice in both experiments. Of note, transgenic mice developed a relapse in both experiments, which was either not observed in wild type mice (second experiment) or the clinical score during the relapses remained significantly lower than in transgenic mice (first experiment). Histological examination of brains 55 days after immunization showed a quantitative difference in mononuclear cells (T- and B- lymphocytes, macrophages), however the localization of the infiltrating cells and demyelination was similar, mainly in the cerebellum and spinal cord (data not shown).



Mouse Genotype	Incidence (%)	Mean day of disease onset (± SEM)	Mean maximum clinical score (± SEM)	Mean accumulative score (± SEM)
Controls	4/4 (100)	13,0 ± 1,3	2,23 ± 0,23	39,35 ± 12,64
LTtg	4/4 (100)	9,5 ± 0,5*	3,15 ± 0,05**	84,2 ± 8,35*

* p < 0.05; ** p < 0.01

Mouse Genotype	Incidence (%)	Mean day of disease onset (± SEM)	Mean maximum clinical score (± SEM)	Mean accumulative score (± SEM)
Controls	7/8 (87,5)	14,5 ± 1	2,1 ± 0,4	29,4 ± 8,9
LTtg	4/4 (100)	6,5 ± 1***	3,4 ± 0,1*	65,9 ± 6,2*

* p < 0.05; ** p < 0.01

Figure 42. Active EAE in *Tg(GFAP-Lta,Ltb)#26* mice. Transgenic overexpression of LTαβ (circles) predisposes mice to an earlier disease onset and significantly higher maximum and accumulative clinical scores in two independent experiments compared to wild type littermates (squares). Furthermore all *Tg(GFAP-Lta,Ltb)#26* mice developed a relapse which was not observed in wild type animals.

3.3.7. Double transgenic 2D2/GFAP-LT mice display early spontaneous neurological symptoms

Despite of the wide use of the EAE model, the factors determining onset, course, distribution, and cellular composition of autoimmune lesions in MS remain largely unknown. In particular, it is unclear which processes are involved in triggering the disease and which drive inflammation during the further course of the disorder. Our unawareness to these vital issues is largely due to the lack of suitable animal models that would develop human inflammatory demyelinating disease spontaneously and reproduce its essential clinical and molecular aspects (Krishnamoorthy et al., 2006). The important unresolved question is how self-reactive cells are "spontaneously" activated. Some investigators link the trigger of MS to microbial infection or molecular mimicry (Kurtzke, 1993), the opposite has been proposed as well, namely that the susceptibility to autoimmune and allergic diseases is related to the decreasing prevalence of infections. Here I sought to investigate if the inflammatory environment developed in *Tg(GFAP-Lta,Ltb)* mice contributes to the activation or to the migration of autoreactive T-cells in the CNS. Therefore I intercrossed *Tg(GFAP-Lta,Ltb)* mice with mice bearing MOG-specific T-cell receptor (TCR) transgenic T-cells (2D2). A large proportion (~50%) of 2D2 mice develops spontaneous optic neuritis, without evidence of clinical or histological signs of EAE. However, only approximately 4% of the 2D2 mice develop spontaneous inflammatory demyelinating disease in the CNS at the age of 2-2,5 months (Bettelli et al., 2003) . Surprisingly 2D2/GFAP-LTab mice showed a very early (28-35 days postnatal) onset of symptoms like limp tail and hind leg weakness (**Figure 43A**), reminiscent to EAE. The observed phenotype progressed within 7 days into complete hind limb paralysis accompanied by significant weight loss. No relapse was observed in the disease course; therefore animals were sacrificed when reaching experimental end point criteria (clinical score ≥ 3 persist for 3 days and 15% weight loss) (**Figure 43B**). Disease incidence was 100% among the double transgenic mice, conversely single transgenic 2D2 or *Tg(GFAP-Lta,Ltb)* mice remained free of clinical signs during the observation period.

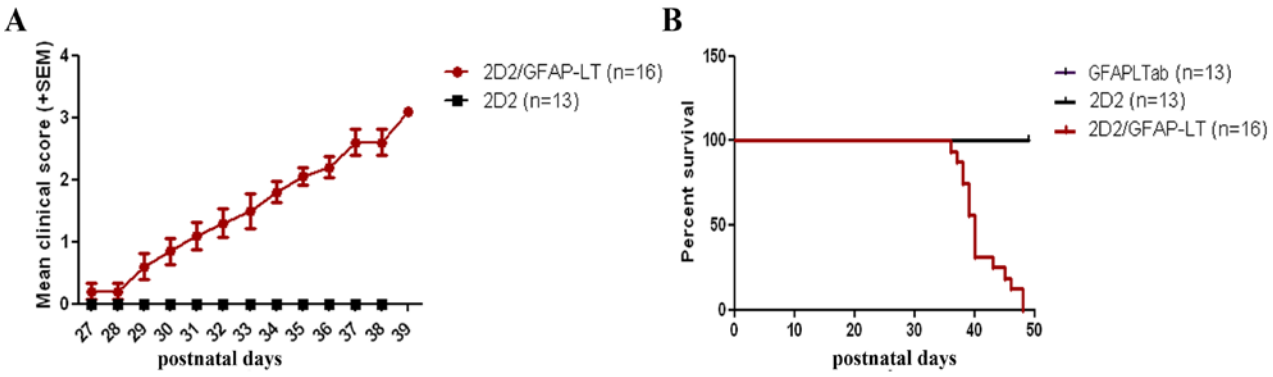


Figure 43. Disease kinetics of spontaneous inflammatory demyelinating disease in double transgenic 2D2/GFAP-LT mice. (A) Spontaneous incidence of early EAE-like symptoms were observed in double transgenic 2D2/GFAP-LT mice housed under SPF conditions (red line, n=16). The disease progressed quickly to severe (clinical score>3) symptoms without remission. Single transgenic 2D2 littermates did not show any clinical symptoms within the observed time frame (black line, n=13). **(B)** Relative time of survival (%) of 2D2/GFAP-LT mice (red line n=16) compared to single transgenic littermates (black and blue lines, n=13). Double transgenic mice succumb to death in 100% while single transgenic littermates remain symptom free.

3.3.8. Histopathological analysis of CNS lesions

Since the 2D2/GFAP-LT mice spontaneously develop neurological symptoms similar to those observed in EAE, I examined tissue sections from the brain and spinal cord of double transgenic mice to assess if they also develop an inflammatory demyelinating pathology. 2D2/GFAP-LT mice were sacrificed approximately 10 days after the first symptoms appeared, along with single transgenic (2D2 and *Tg*(GFAP-Lta,Ltb)) littermates as controls. Localization and cellular composition of the lesions was determined by immunohistochemical stainings (**Figure 44**). Inflammatory infiltrates were dominated by mononuclear cells. Severe inflammatory cell infiltrations were found in the brain stem meninges (n=13) and cerebellum (n=3) of double transgenic mice (**Figure 44A**). However, the most prominent pathological changes were observed in the spinal cord, especially in the lumbar region (n=16) (**Figure 44B**). Although, the localization of the lesions resembles those usually found in the EAE model, importantly in 2D2/GFAP-LT mice high percent of the infiltrating cells are B-cells, a phenomenon reminiscent to human MS infiltrates, but rarely observed in EAE model in C57BL/6 mice. Besides the B-cells, $CD3^+$ T-cells and $Mac\text{-}3^+$ macrophages were also found in the spinal cord lesion of double transgenic mice, concomitant with profound inflammatory demyelination (LFB) (**Figure 44B and Figure 45**). Age matched single transgenic 2D2 or *Tg*(GFAP-Lta,Ltb) mice did not display any pathological alterations.

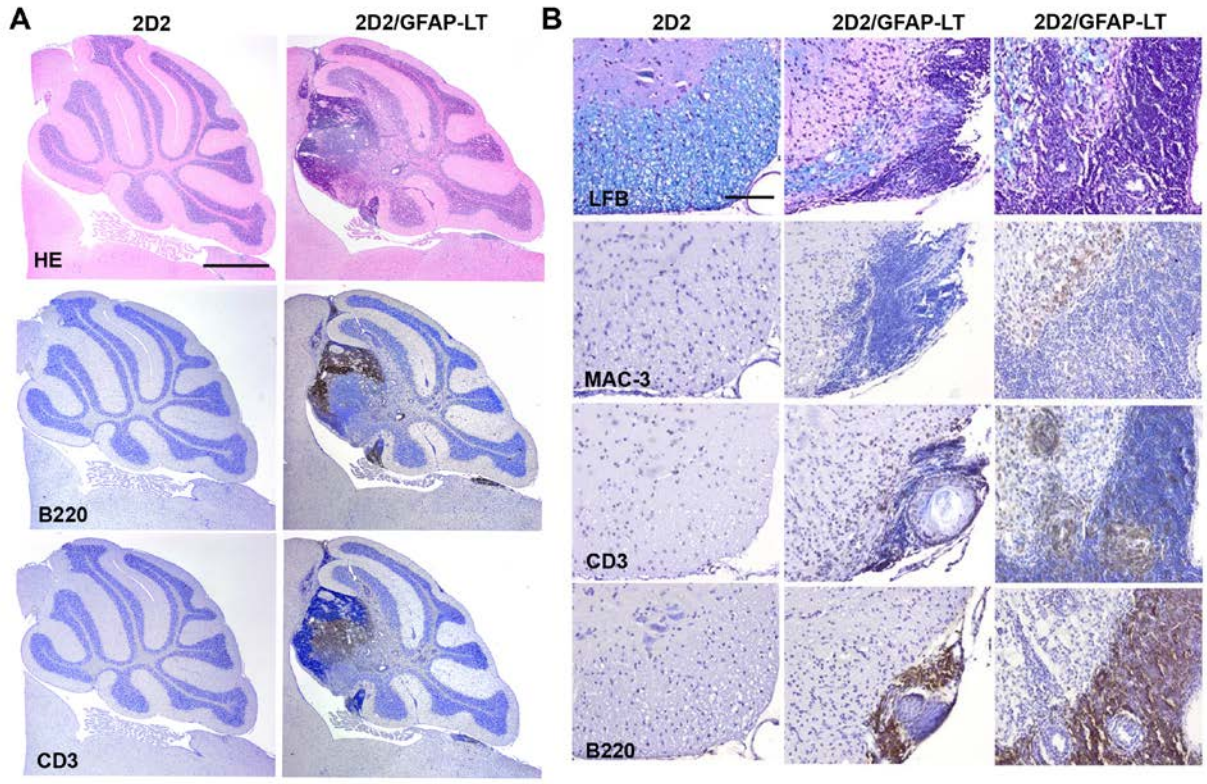


Figure 44. Histological analysis of the CNS from sick 2D2/GFAP-LT mice. (A) Immunohistochemical characterization of the infiltrates in cerebellum and brain stem meninges of double transgenic mice and 2D2 single transgenic littermates. HE, $B220^+$ B-cells and $CD3^+$ T-cells are indicated (scale bar: 500 μm). **(B)** Special stainings in the spinal cord of 2 independent double transgenic mice compared to a single transgenic 2D2 mice. Demyelination is visualized by LFB stainings, infiltrating macrophages (MAC-3), T-cells (CD3) and B-cells (B220) are indicated (scale bar: 50 μm).

The lesions in 2D2/GFAP-LT mice were circumscribed rather than diffuse, and interestingly displayed follicle like structures in brain stem meninges (**Figure 46**) and in the spinal cord (**Figure 47**). A great number of human SPMS cases were reported to contain B-cell follicle like structures in the cerebral meninges, which were identified based on the presence of $Ki67^+/CD20^+$ proliferating B-cells, plasma cells, T-cells and $CD35^+$ FDCs (Howell et al., 2011). To confirm the presence of such follicle-like structures in the spinal cord of 2D2/GFAP-LT mice I analysed spinal cord cross section from 4 independent 2D2/GFAP-LT mice, with a clinical score of 3. Immunohistochemistry identified a high number of B-cells, $CD4^+$ T-cells, however only very few $CD8^+$ T-cells (**Figure 47**).

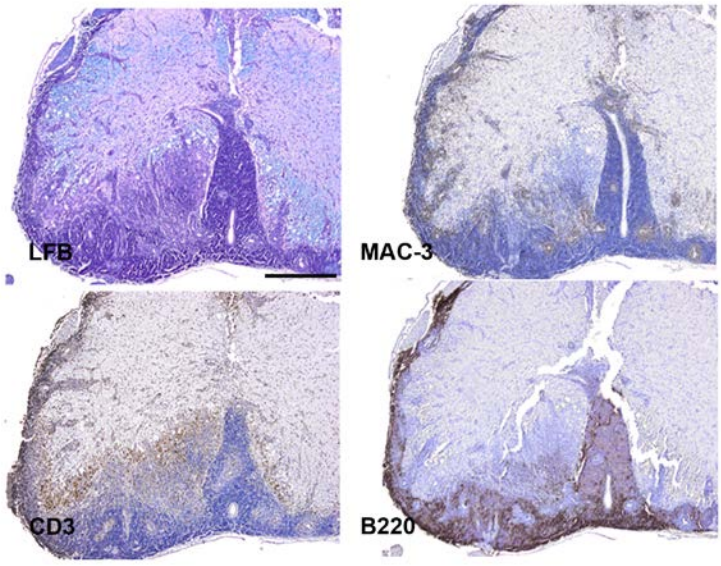


Figure 45. Spinal cord cross sections in 2D2/GFAP-LT mice. Cross sections of the spinal cord from 2D2/GFAP-LT mouse, showing the extent of demyelinated area (LFB) and inflammatory infiltrates. Macrophages (MAC-3), T-cells (CD3) and B-cells (B220) are indicated (scale bar: 500 μm).

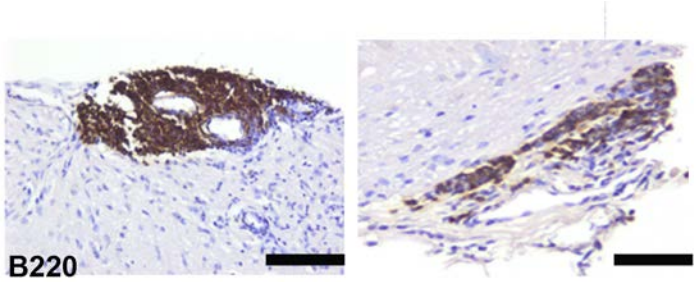


Figure 46. B-cell infiltrates in the brain stem meninges of 2D2/GFAP-LT mice. $B220^+$ B-lymphocytes in two independent double transgenic mouse with clinical score 3 localized in the brain stem meninges.

The low frequency of $CD8^+$ T-cells is characteristic for the 2D2 transgenic mice. 2D2 mice show a skewing toward $CD4^+$ T-cells in spleens, as well as the CD4/CD8 single positive ratio in thymus is biased toward $CD4^+$ compartment (Bettelli et al., 2003). Furthermore I observed $CD21/35^+$ germinal center B-cells as well as FDC networks in the infiltrates, which corroborates the development of ectopic lymphoid follicles in 2D2/GFAP-LT mice. None of the single transgenic parental lines displayed follicle-like infiltrates in the CNS.

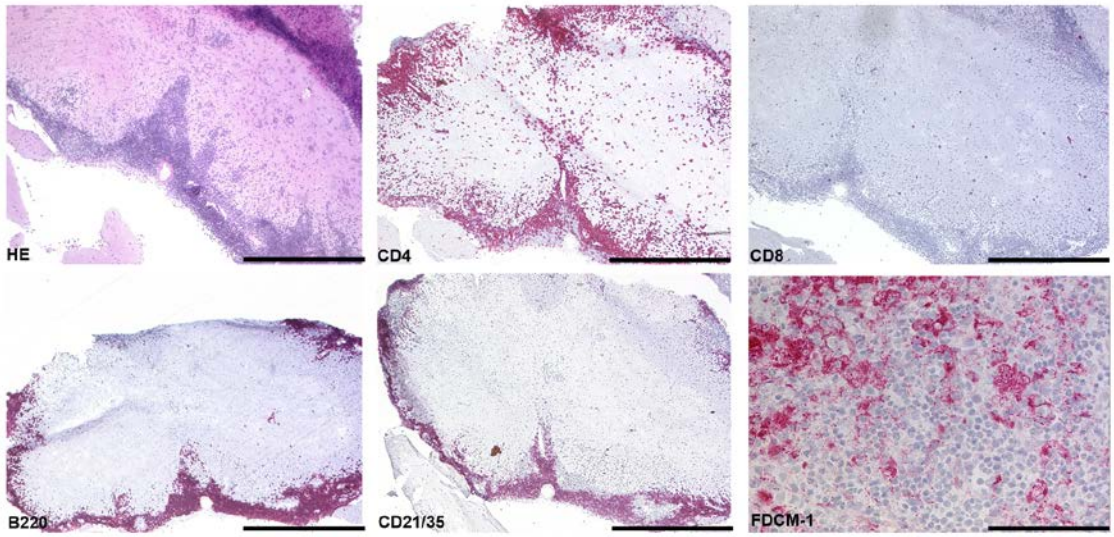


Figure 47. Histological analysis of inflammatory infiltrates in the spinal cord of 2D2/GFAP-LT mice.
H/E, $CD4^+$ T-cells, $CD8^+$ T-cells, $B220^+$ B-cells, $CD21/35^+$ germinal center B-cells (scale bar: 500 μm) and FDC-$M1^+$ follicular dendritic cells and networks are indicated (scale bar: 100 μm).

3.3.9. Quantification of immune cells within the CNS lesion in 2D2/GFAP-LT mice

To gain a quantitative and qualitative overview of the immune cell populations in the spinal cord of sick 2D2/GFAP-LT mice, age matched wild type and single transgenic 2D2 and *Tg*(GFAP-Lta,Ltb) mice, we performed flow cytometry on Percoll gradient separated infiltrate cells. Large numbers of inflammatory cells were recovered from the spinal cords of sick 2D2/GFAP-LT mice in contrast to healthy 2D2 and *Tg*(GFAP-Lta,Ltb) single-transgenic animals, which yielded only very few inflammatory cells (**Figure 48**). Consistent with the histological evaluation, a drastic increase in lymphocytes, predominantly in $CD4^+$ T-cells, B-220^+ B-cells and regulatory T-cells was observed, along with significantly elevated granulocyte ($CD11b^+Ly6G^+$) and inflammatory monocyte ($CD11b^+Ly6C^{hi}$) population. Of note, double transgenic animals displaying less severe (clinical score 2) symptoms were also analysed and included in the quantification, which most likely explains why the particular subsets of lymphocytes did not reach statistical significance. However, a clear trend towards increased numbers of $CD4^+$ T-cells, B-220^+ B-cells can be seen.

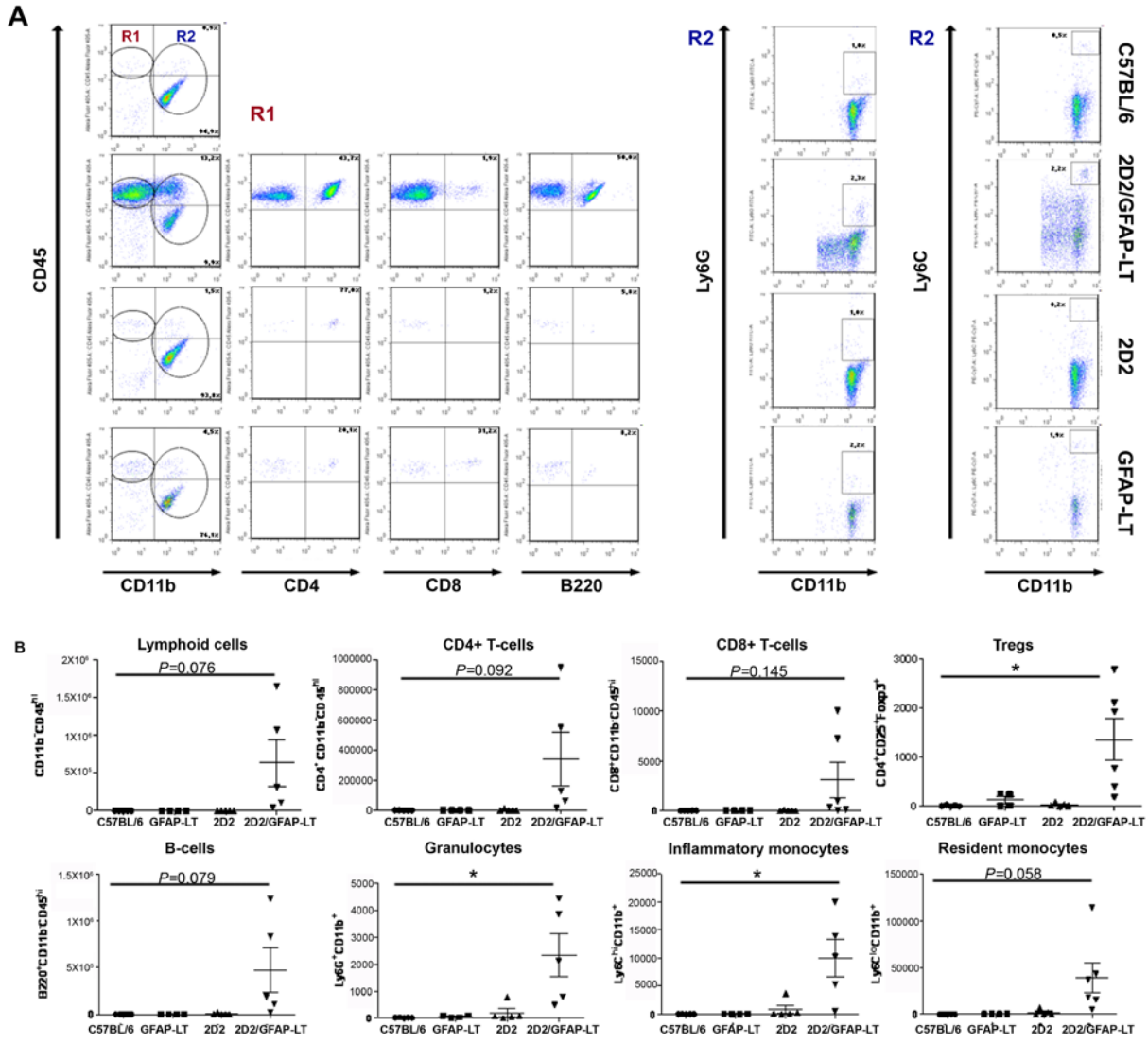


Figure 48. Flow cytometric analysis of CNS infiltrating mononuclear cells. (A) CNS mononuclear cells isolated by Percoll gradient centrifugation from sick 2D2/GFAP-LT mice, age matched wild type and single transgenic 2D2 and *Tg*(GFAP-Lta,Ltb) mice were stained with CD45, CD11b. Lymphocytic population was identified as R1 ($CD45^{hi}$, $CD11b^{-}$) and CD4, CD8, B220, CD25, FoxP3 expression was analysed in the gated R1 population. Monocyte population was classified as R2 ($CD11b^{+}$) where Ly6C and Ly6G was further analysed. **(B)** Quantification of total cell count of $CD4^{+}$, $CD8^{+}$, $B220^{+}$ lymphocytes, Regulatory T-cells, and $CD11b^{+}Ly6G^{+}$ granulocytes, $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes and $CD11b^{+}Ly6C^{low}$ resident monocytes in sick 2D2/GFAP-LT mice, age matched wild type and single transgenic 2D2 and *Tg*(GFAP-Lta,Ltb) mice. *: $P<0.05$;

3.3.11. Migration of immune cells from the spleens of sick 2D2/GFAP-LT mice

To investigate macroscopic alterations outside of the CNS in double transgenic mice, I first evaluated the size of the spleen. Interestingly spleens from 2D2/GFAP-LT appeared to be smaller than spleens from the single transgenic 2D2 littermates. This drastic decrease in the

size of the spleen was reflected by a highly significant reduction in the number of splenocyte measured by flow cytometry (**Figure 49**). This finding implies that T-cells bearing MOG specific T-cell receptors might migrate directly from the spleen to the CNS.

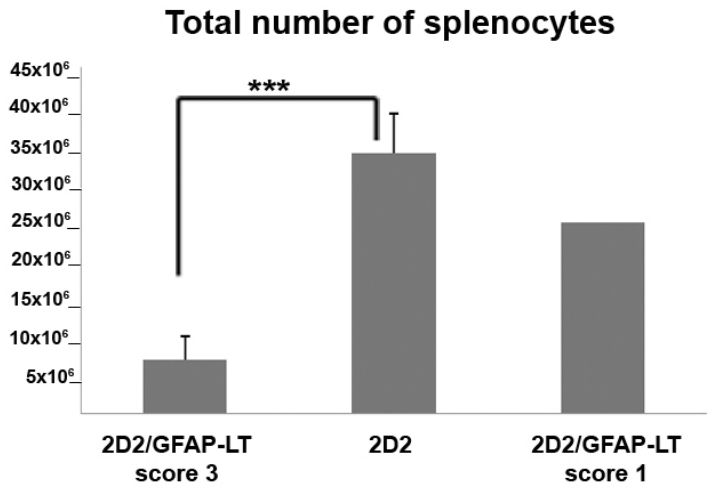


Figure 49. Significant reduction in total number of splenocytes in double transgenic mice. The observed decrease in the size of the spleen in 2D2/GFAP-LT animals was corroborated by flow cytometric analysis of the total number of splenocytes. Double transgenic mice with clinical score display significant decrease in the number of splenocytes compared to 2D2 single transgenic littermate.

3.4. Discussion

Multiple sclerosis is a complex immune mediated disease of the central nervous system. Its aetiology is currently unknown and its pathogenesis is only partly understood (Lassmann and van Horssen, 2011). Active MS lesions display inflammatory changes suggestive of a combined attack by autoreactive T- and B-cells against the CNS (Franciotta et al., 2008). Previous studies using various knock out models of the LTβR-signalling as well as pharmacological blocking of LTβR (LTβR-Ig) proposed an important role for LT in demyelinating processes, most notably in the pathogenesis and progression of multiple sclerosis. To elucidate the contribution of lymphotoxin to CNS inflammation and demyelination I generated a transgenic mouse model with astrocyte specific overexpression of LTα and LTβ. *Tg*(GFAP-Lta,Ltb) mice develop neuroinflammation, presented as prominent astrogliosis and microgliosis concomitant with numerous T-cell infiltration already at the age of eight-twelve weeks. The blood brain barrier was shown to be intact in transgenic mice. Already at the age of three months significant up-regulation of pro-inflammatory (*CXCL1*, *CXCL9* and *CXCL10*) and homeostatic chemokines (*CXCL13*, *CCL19*) and adhesion molecule (*VCAM*) was observed on mRNA and partially on protein level. Mice were followed up histologically until 12 months of age. Importantly, *Tg(GFAP-Lta,Ltb)* mice when immunized with MOG 35-55 developed a significantly earlier disease onset and more severe disease manifestation compared to wild type littermates. Of note, within the investigated timeframe *Tg(GFAP-Lta,Ltb)* mice did not develop any signs of spontaneous demyelination and autoimmunity.

Spontaneously developing organ-specific autoimmune disease models in rodents are rare. The goal of the new generation mouse models is not only to closely resemble human disease clinically and histologically but also to develop spontaneously without artificial manipulations such as immunization or lymphocyte transfers (Wekerle et al., 2012). So far the best example for such a model was the NOD mouse model, which fulfils these requirements for the study of human autoimmune diabetes mellitus. Furthermore the *Tg(Ela1-Lta,b)* mice, I presented in my thesis represents a novel mouse model mimicking the spontaneous development of human AIP. Spontaneous autoimmune disease was also reported in the CNS. Several different TCR transgenic models with spontaneous EAE-like symptoms have been reported over the past years. Most of these are based on $CD4^+$ T cells that recognize myelin basic protein (MBP) (Lafaille et al., 1994) (Goverman et al., 1993), myelin proteolipid protein (PLP) (Waldner et

al., 2000), or MOG (Bettelli et al., 2003) (Anderson et al., 2012; Pollinger et al., 2009) on different genetic backgrounds. Low frequency (<15% under conventional and 0% under SPF conditions) of spontaneous disease development was observed in mice with a TCR specific for MBP in the context of I-Au (Goverman et al., 1993) (Lafaille et al., 1994), and MOG specific TCR transgenic mice on C57BL/6 background (2D2) (Bettelli et al., 2003). The frequency increased in the absence of regulatory lymphocytes in these mice when intercrossed with Rag1 deficient mice (Lafaille et al., 1994). Remarkably, mice expressing PLP specific TCRs on CD4+ T-cells on SJL background develops spontaneous EAE in 40-67% (Waldner et al., 2000) on SPF background without any further intercrossing. Although these models have contributed greatly to our understanding on the role of $CD4^+$ T cells in CNS autoimmunity, increasing evidence suggests that $CD8^+$ T cells and B-cells are also important in the pathogenesis of MS. The first TCR transgenic mice that harbour both myelin-reactive $CD4^+$ and $CD8^+$ T on NOD background (1C6) (Anderson et al., 2012) display signs of spontaneous disease development in approx. 1%. To study the immune response and development of disease in hosts that have $CD8^+$ T and/or $CD4^+$ cells as well as B-cells that recognize the same myelin antigen (MOG), 2D2 and 1C6 mice were crossed to IgH_{MOG} knock-in mice. If MOG reactive $CD4^+$ T cells (from 2D2 TCR Tg) and MOG-reactive B cells (from IgH_{MOG} knock-in) coexist on the C57BL/6 background, the mice develop spontaneous disease with 50% incidence and exhibit a distinct pathological pattern in which lesions are localized mostly in the spinal cord and optic nerve (Bettelli et al., 2006) (Krishnamoorthy et al., 2006). Moreover, those mice exhibit development of ectopic lymphoid follicle-like structures in the spinal meninges. Notably, 1C6 x IgH_{MOG} mice developed spontaneous disease with 45-79% incidence with an average age of onset of 15 weeks (Anderson et al., 2012).

The stimuli triggering MS pathogenesis has been commonly attributed to environmental factors, in particular microbial infection (Ascherio and Munger, 2007). To this end, a study with the relapsing-remitting model of EAE (SJL/J mice with increased proportions of MOG-specific autoimmune $CD4^+$ T-cells simultaneously develop a relapsing-remitting EAE) (Pollinger et al., 2009) has established that CNS autoimmune T-cells are activated and become pathogenic after interaction with the healthy, commensal gut flora (Berer et al., 2011). Apart from activation by molecular mimicry autoreactive T-cells could also be stimulated by the exposure of high local concentrations of cytokines (Hohlfeld, 1997). Therefore, I utilized the novel *Tg(GFAP-Lta,Ltb)* mouse model with CNS specific inflammation and intercrossed with mice bearing transgenic T-cells expressing MOG specific

T-cell receptors (2D2). The 2D2/GFAP-LT mice developed a very early onset of spontaneous inflammatory demyelinating disease. The incidence of the neurological symptoms, with ataxia and classical paralysis was 100%. Histological and flow cytometry analysis confirmed that double transgenic 2D2/GFAP-LT mice develop severe inflammatory cell infiltration (predominantly $CD4^+$ T-cells, B-220^+ B-cells and Mac-3^+ macrophages) concomitant with prominent demyelination primarily in the cerebellum, brain stem and spinal cord. The inflammatory infiltrates resemble tertiary lymphoid tissues, which can also be found mostly in the meninges of human MS patients (Prineas, 1979) (Howell et al., 2011). In this novel 2D2/GFAP-LT substantial amount of infiltrating inflammatory cells are B-cells, which are only rarely observed in active EAE models or in the above discussed spontaneous EAE-like models. This allows us in the future, to investigate the antibody-independent role of B-cells in the disease pathogenesis.

Here I hypothesized that auto-reactive MOG specific T-cells get activated in the peripheral lymphoid tissues. In response to a cytokine and chemokine gradient resulting from the expression of lymphotoxin and its downstream targets, these activated autoreactive T-cells will migrate to the CNS. Thus, astrocyte derived LT induces a local inflammatory environment favourable of evolving autoimmune responses. As the next step, MOG specific T-cells get reactivated in the CNS upon meeting their target antigen, which triggers the further release of cytokines and soluble mediators. These processes contribute to the disruption of the BBB, and stimulate chemotaxis, resulting in a second wave of inflammatory cell recruitment. This time also antigen specific B-cells will migrate to the CNS and elicit multifaceted functions, either in T-cell activation or antibody production, causing disease exacerbation. Factors that attract B-cells are likely the homeostatic chemokines (e.g. CXCL13 and CCL19) whose production is most likely triggered by LT in the GFAP/2D2 system. This would imply to a role for LT in disease relapses.

The second possibility is that autoreactive T-cells get activated within the CNS. Resting auto-reactive T-cells may leave small blood vessels in the CNS at a very low rate. In the 2D2/GFAP-LT model the number of attracted antigen-specific T-cells is greater than in the single transgenic 2D2 mice, consequently the chance that a MOG-specific CD4 T-cell reaches the parenchyma of the brain is much higher in the 2D2/GFAP-LT transgenic mice. If they recognize an antigen presented by the brain's resident antigen-presenting cells, they might become activated within the CNS, which results in local clonal expansion and recruitment of further inflammatory cells, including self-antigen specific B-cells. Due to the higher LT

concentration and the presence of lymphoid chemokines (CXCL13 and CCL19) the infiltrating cells form ectopic lymphoid follicles.

This is the first study to our knowledge that describes the role of local CNS immunity in attracting myelin specific T-cells, facilitating to study the initiation and pathogenesis of a complex spontaneous demyelinating autoimmune disease.

3.5. Outlook

(1) First of all, I plan to intercross the intermediate LT expresser transgenic line (GFAP-Lta,Ltb#23) with the 2D2 transgenic mice, to elucidate the disease pathogenesis, e.g. the disease onset and the localization of the possible lesions. The aim is to see if lower level and different distribution of LT expression would alter the development of spontaneous inflammatory demyelinating disease.

(2) According to the hypothesis, autoreactive T-cells in 2D2/GFAP-LT mice can also get activated inside of the CNS. Therefore, we would like to analyse with flow cytometry the activation status of $CD4^+$ T-cells in the CNS (CD62L, CD25, CD44) in the spleen and in the cervical LNs (CNS draining peripheral lymph nodes). Alternatively to the hypothesis I proposed in my discussion, it is well possible that B-cells arise before transgenic T-cells infiltrate the CNS. Therefore we would like to perform a time-course analysis on day 21 and day 30 post natal and investigate lymphocyte subpopulations by immunohistochemistry and flow cytometry.

(3) Next, I would like to determine the $CD4^+$ T helper cell (Th) polarization pattern underlying the spontaneous disease in double transgenic mice. Th cells can be classified into subsets (Th1, Th2, Th17 and T_{reg}) based on their distinct cytokine expression and master transcription factors that are required to linage commitment. Therefore, I will analyse these cytokines and transcription factors by RT-PCR, in the spinal cord and spleen of double transgenic mice as well as in 2D2 and *Tg*(GFAP-Lta,Ltb) single transgenic littermates for control.

(4) To describe the inflammatory cells in more detail I also plan to characterize the specific B-cell receptors and T-cell receptors on the infiltrating cells in the CNS.

(5) Increased intrathecal production of immunoglobulins in the CSF is observed in more than 90% of MS patients. These Igs include IgG, IgM, IgD, and IgA. Concurrent elevations of Igs are not observed in the serum, indicating that the immunoglobulins are produced locally within the CNS. Cerebrospinal IgG and IgM usually demonstrate an oligoclonal pattern when the proteins are separated by electrophoretic methods (Cross et al., 2001). In sick double transgenic mice, I will test for the presence of the various immunoglobulin subclasses and oligoclonal IgG in brain homogenates. Furthermore, I would like to test sera of sick 2D2/GFAP-LT as well as 2D2 and *Tg*(GFAP-Lta,Ltb) mice to confirm if anti-MOG antibodies have undergone class switch from IgM to IgG.

(6) To examine the importance of B-cells in the development and course of spontaneous inflammatory demyelinating disease, I set out to deplete B-cells in the 2D2/GFAP-LT model with a monoclonal anti-mouse CD-20 antibody. Moreover, I also plan to administer LTβR-Ig to investigate if blocking LTβR-signalling would delay or alter the course of spontaneous disease development. First both treatments will be given prophylactically; Mice will be treated starting from day 20 (approximately 10 days before onset), on a weekly basis with either LTβR-Ig or anti CD-20 antibody. In case this treatment induces a beneficial effect in double transgenic mice, we plan to repeat the treatment in a more physiological setting, namely to start administering the drug once the symptoms have become clinically apparent (clinical score 1). Treatment efficacy will be monitored in case of anti CD-20 antibody by quantifying B-cells in the peripheral blood. To monitor LTβR-Ig treatment, absence of germinal centers and FDC-networks will be confirmed in spleens.

(7) Furthermore, I would like to establish the passive EAE protocol (adoptive transfer of T-cells from MOG TCR transgenic (2D2) mice, stimulated with MOG). I plan to use this model in *Tg*(GFAP-LTa,LTb) mice on $Rag1^{-/-}$ background. This would enable me to elucidate the importance of B-cells in the initiation of EAE, given that the only lymphocytes present in this setting will be the auto-reactive T-cells.

(8) In order to examine the role of LTβR on specific brain cells, and to define the cell type that responds to LT, I intercrossed *Tg(GFAP-LTa,LTb)* mice to $LT\beta R^{loxP/loxP}$ mice in combination with using the oligodendrocyte specific Cre-deleter line (MOGi-Cre) as well as the neuron specific Nestin-Cre line. Here, my aim is to investigate whether the direct cross-talk between astrocyte derived lymphotoxin and oligodendrocytes or neurons could contribute to the observed astrogliosis and microgliosis in *Tg(GFAP-LTa,LTb)* mice.

(9) Additionally, I would like to induce EAE in mice lacking LTβR on either oligodendrocytes or neurons, to test the possible importance of LTβR locally in the CNS in EAE development.

3.6. Material and Methods

Generation of Tg(GFAP-Lta,Ltb) mice : The Ela promoter from the *ELa1-Lta,b* expression constructs was cut out by Not I, BamH I double digest and the remaining DNA expression vector cassettes purified (LTa-expression cassette; LTb expression cassette). The gfa2-lacZ plasmid was used as template to amplify the promoter sequence. This plasmid is a pUC vector containing the human GFAP sequences from -2163 to +47 (the human *gfa2* segment), with the initiating ATG mutated to TTG, placed in front of the standard, cytoplasmic, E. coli *lacZ* gene, which in turn is followed by a fragment of the mouse protamine-1 (mP-1) gene (Brenner et al., 1994). The latter supplies an intron, stabilizing 3' UTR, and a polyadenylation signal. The *lacZ* gene was excised by digestion with BAMH I, retaining the mP-1 segment.
The fwd primers for both LTa and LTb were designed with a 24 base overhang to create a BamH I cut site. The PCR products of LTa and LTb were ligated separately to the gfa2 promoter using a T4 DNA Ligase (New England Biolabs). The positive clones were selected and analyzed by sequencing (Microsynth). Bacterial colonies containing the GFAP-LTa and GFAP-LTb constructs were then picked, cultivated and screened by digestion with BamHI. After sequencing, plasmids and subsequent linearization microinjection was performed into pronuclei of C57BL/6N zygotes with equimolar ratio of independent, linearized constructs harbouring each the *Lta* and *Ltb* murine cDNA.
Upon PCR analysis, from 35 born offspring, 3 were identified harbouring both LTa and LTb transgenes. All three potential founder lines expressed the transgenes on mRNA level in the brain and spinal cord.

Evans Blue: Evans blue (400 ml 1% Evans blue dye in PBS) was intravenously administered. 4 hours later mice were euthanized, and intracardiac perfusion was performed through the left ventricle with cold PBS to remove intravascular albumin-Evans blue. Brains and spinal cords were removed. Pictures were obtained with a Canon Power shot G6 camera. As a positive control, mice, in which a mechanical lesion was set in the cerebrum to disrupt the blood brain barrier was used.

RNA isolation from CNS tissue: Mouse tissue or cells were rapidly dissected, snap frozen in isopentane, and stored at −80 °C. Total RNA was prepared according to the TRIzol method (Invitrogen).

Real-time polymerase chain reaction: For mRNA expression analysis Real-time PCR was performed using Fast Start SYBR Green Master Rox (Roche). Primers were custom made by Microsynth. Real-time PCR was performed on an ABI PRISM 7900 HT Fast Real-Time PCR System (AB). Data were generated and analyzed using SDS 2.4 and RQ manager 1.2 software. The mRNA expression levels were normalized to the housekeeping gene *Gapdh.*

Cytokine Protein array and Immunoglobulin measurement: Cytokine protein levels from mouse CNS tissues were measured using a multiplexed particle-based flow cytometric cytokine assay (Vignali et al., 2000). Kits were purchased from BioRad (Ismaning, Germany) and R&D Systems (Oxon, UK) and Millipore (Zug, Switzerland). The procedures closely followed the manufacturer's instructions. The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus, Millipore, Zug, Switzerland) Multiplexed particle-based flow cytometric assays. Protein concentration from CNS homogenates were determined by BCA (explain, vendor) assay, and final cytokine concentrations were normalized to total protein concentrations.

Induction of EAE: Female 8–10 weeks old mice from each group were immunized subcutaneously with 200 µg of $MOG_{35–55}$ peptide emulsified in complete Freund's adjuvant containing 1 mg of *Mycobacterium tuberculosis* (H37RA, Difco Laboratories). The mice received intraperitonal injections of 250 ng pertussis toxin (List Laboratories) at the time of immunization and 48 h later. Animals were observed daily and neurological deficits were quantified on an arbitrary clinical scale: 1, limp tail; 2, hind limb weakness or abnormal gait; 2.5: unilateral hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete bilateral hind limb paralysis and partial fore limb paralysis 4, moribund, complete paralysis; 5, death.

CNS Mononuclear Cell Isolation: At the end stage of disease, mice were anesthetized, perfused with 20 mL of ice cold PBS. Spinal cord tissue samples were collected and homogenized mechanically in dissecting medium (50 ml 1x HBSS, 650 µl 45% Glucose, 750 µl 1M HEPES). After centrifugation (300xg, 10 min) pellet was resuspended in 70% Sucrose. Mononuclear cells were collected from the 35%/30% interface of a Percoll gradient after centrifugation at 450×g for 25 min at 4°C. Collected cells were washed once with FACS buffer (10 ml FCS, 10 ml 5M EDTA pH8) and stored on ice for further use.

Flow Cytometry: Fluorescence staining of cell samples was performed using the following mouse specific antibodies: anti-CD45-pacific blue, anti-CD11b-FITC/APC, anti-B220-Biotin, anti-CD4- PerCP, anti-CD8a-PE, anti-Ly6C-PE-Cy7, anti-CD115-PE, anti-Ly6G-FITC, anti-CD25-APC and the strepavidin-APC complex (all eBioscience). Intracellular staining of Foxp3 was performed with the corresponding staining kits (eBioscience) according to the manufacturer's protocol. Samples were acquired using a Canto II flow cytometer (BD) and data analyzed using FlowJo (TreeStar).

Histology: For histological analysis, spinal cords were dissected, tissues were fixed either in 4% buffered formalin fixed and embedded in paraffin or in snap frozen in OCT media. For immunohistochemical analysis of spinal cord tissues, we used monoclonal rat anti-mouse MAC3 (clone M3/84; BD Biosciences), monoclonal rat anti-human/mouse CD3 (clone CD3-12; Serotec), monoclonal rat anti-mouse B220 (clone RA3-6B2; Serotec), and monoclonal mouse anti-amyloid precursor protein (clone 22C11; Chemicon), Luxol fast blue, anti-AQP4 (rabbit polyclonal; Sigma, Germany) and anti-GFAP FDCM-1 (BD Biosciences cat. Nr. 551320), anti-CD21/35 antibodies (BD Biosciences cat. Nr. 553817 rat monoclonal (7G6)).

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5. Curriculum Vitae

PERSONAL DATA:

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EDUCATION:

1987-1995:	Dienes Valeria Altalanos Iskola, Grundschule, Szekszard, Hungary
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1999-2005:	M.S. Environmental Engineering, Budapest University of Technology and Economics (BUTE), Faculty of Chemical Engineering
2004:	Vienna University of Technology, Faculty of Environmental Chemistry
2003-2005:	BUTE Department of Environmental Biotechnology – Diploma Thesis – Monitoring of specific growth speed of nitrifying bacteria**.** Research leader: Dr. Andrea Jobbagy
2005:	Centro Universitario Mauà, Sao Paulo, Brazil – Research on biological waste water treatment, studying the effect of organic loading on nitrifying and denitrifying bacteria.
2003-2006:	M.S. Biomedical Engineering, BUTE
2005-2006:	BUTE Department of Inorganic Chemistry – Diploma Thesis – Development of silicone based implants (e.g. polymer stents). Research leader: Dr. Jozsef Nagy
2006-2007:	Genzyme Corporation, Cardiovascular research-cardiac cell therapy group, Framingham, MA-USA
2007-2008:	SOLVO Biotechnology Inc. Szeged-Hungary Utilization of ABC transporter assays in drug discovery and development.
2008 –present:	PhD thesis at the Institute of Neuropathology, University Hospital Zurich, Switzerland; Research leader: Prof. Dr. Mathias Heikenwälder. Title: Ectopic lymphotoxin expression in the pancreas and in CNS predisposes for autoimmunity.

6. Publications and awards

Factors Affecting Residence Time of Mesenchymal Stromal Cells (MSC) Injected into the Myocardium

Jason Westrich[1], Peter Yaeger[1], Chufa He[1], Jeff Stewart[1], Raymond Chen[1], Gitta Seleznik[2], Shane Larson[3], Bruce Wentworth[1], Sam Wadsworth[1], Geoffrey Akita[1], Gyongyi Molnar[1]

Cell transplantation – 2010;19(8):937-48.

The unexpected role of lymphotoxin signaling in cancer development: From lymphoid tissue formation to liver and prostate cancer development.

Wolf MJ, Seleznik GM, Zeller N, Heikenwalder M

Oncogene – 2010 Sep 9;29(36):5006-18

TAK1 suppresses a NEMO-dependent, but NF-κB-independent pathway to liver cancer

Bettermann K, Vucur M, Haybaeck J, Koppe C, Janssen J, Heymann F, Weber A, Weiskirchen R, Liedtke C, Gassler N, Müller M, de Vos R, Wolf MJ, Boege Y, Seleznik GM, Zeller N, Erny D, Fuchs T, Zoller S, Cairo S, Buendia M, Prinz M, Akira S, Tacke F, Heikenwalder M, Trautwein C, Luedde T

Cancer Cell. 2010 May 18;17(5):481-96.

Lymphotoxin's link to carcinogenesis: Friend or Foe?

From lymphoid neogenesis to hepatocellular carcinoma and prostate cancer

Wolf MJ, Seleznik GM, Heikenwalder M.

Adv Exp Med Biol. 2011;691:231-49.

MANUSCRIPT IN PREPARATION:

A Lymphotoxin driven, IL17-independent pathway to autoimmune pancreatitis

Gitta M. Seleznik, Theresia Reding, Franziska Romrig, Yasuyuki Saito, Alexander Mildner, Stephan Segerer, Li-Kang Sun, Stephan Regenass, Maciej Lech, Hans-Joachim Anders, Donal McHugh, Teru Kumagi, Yoichi Hiasa, Carolin Lackner, Johannes Haybaeck, Eliane Angst, Aurel Perren, Maria Luisa Balmer, Emma Slack, Andrew MacPherson, Markus Manz, Achim Weber, Jeffrey Browning, Melek Canan Arkan, Thomas Rülicke, Adriano Aguzzi, Marco Prinz, Rolf Graf* and Mathias Heikenwalder*.

Submitted to Journal of Experimental Medicine, March 2012

T cell-derived Lymphotoxin is sufficient for nasal-associated lymphoid tissue microarchitecture and adaptive immunity

Nicolas Zeller*, Javier Rangel-Moreno*, Maria de la Luz Garcia-Hernandez, Hauke Schmidt, Gitta Seleznik, Claudia Detje, Ulrich Kalinke, Adriano Aguzzi, Troy Randall*, Mathias Heikenwalder*, Marco Prinz*

Submitted to Journal of Immunology, January 2012

Soluble LTα_3 produced by innate lymphoid cells regulates IgA production in the gut

Andrey A. Kruglov[1,2], Dmitry V. Kuprash[2], Sergei I. Grivennikov[3], Gitta M. Seleznik[4], Sandra Prepens[1], Mathias Heikenwälder[4,5], Dan R. Littman[6], Alexey Tumanov[7], Sergei A. Nedospasov[1,2]

In preparation for Nature Immunology

NIK (NF-kB-Inducing Kinase) acts as a pro-death kinase downstream of TNFR1

Layla Boutaffala [1], Mathieu Bertrand [3], Caroline Remouchamps [1], Gitta Seleznik [4], Cécile Benezech [5], Sandrine Marchetti [6], Tracy Jay [4], Corinne Ganeff [1], Jean-Ehrland Ricci [6], Jacques Piette [2], Peter Vandenabeele [3], Jorge Caamano [5], Mathias Heikenwalder [4], Emmanuel Dejardin [1,]

In preparation

AWARDS:

Poster Prize – 9th Day of Clinical Research 8.04.2010 – University Hospital Zurich

A novel mouse model for autoimmune pancreatitis: Generation of transgenic mice to investigate the mechanisms of chronic pancreatitis induced tissue destruction and carcinogenesis.

Gitta Maria Seleznik[1*], Li Kang Sun[3*], Therersia Reding-Graf[3], Achim Weber[2], Alexander Mildner[4], Stephan Regenass[5], Thomas Rülicke[6], Rolf Graf[3,*], Mathias Heikenwalder[1,*].

EPC Research Prize – Best Presentation – European Pancreatic Club meeting, Stockholm June 16-19 2010

A mouse model for autoimmune pancreatitis: Investigation of the mechanisms driving chronic pancreatitis induced tissue destruction.

Gitta Maria Seleznik[1*], Li Kang Sun[3*], Therersia Reding-Graf[3], Achim Weber[2], Alexander Mildner[4], Stephan Regenass[5], Thomas Rülicke[6], Rolf Graf[3,*], Mathias Heikenwalder[1,*].

7. Acknowledgements

I would like to express my gratitude to Prof. Adriano Aguzzi for having provided me the opportunity to perform my Ph.D. thesis in his institute and for his scientific advice during my PhD thesis.

The driving force behind this work was Prof. Dr. Mathias Heikenwälder with his bright ideas, enthusiasm and constant support. I thank him for the great care that he took in my PhD education, by frequent assessment of my research progress, and by his consistent motivation and guidance. I am grateful for the many opportunities that he created for me: to write research proposals, to collaborate in many exciting scientific studies, and to present my work at international scientific meetings.

I would like to thank Prof. Maries van den Broek and Prof. Michael Detmar for their support and help as members of my PhD committee.

Furthermore, I am very thankful to Prof. Rolf Graf and Theresia Reding Graf for the fruitful and enjoyable collaboration on the autoimmune pancreatitis related experiments in the first chapter. Their scientific but also human advice and encouragement motivated me during the past years.

On the same note, I would like to thank to the collaborators on the autoimmune pancreatitis project from the University Hospital Zurich: Dr. Achim Weber, Dr Stephan Segerer, Dr. Stephan Regenass and Yasuyuki Saito.
I would also like to acknowledge external collaborators, first of all Prof. Dr. Marco Prinz and Stefanie Gaupp from Freiburg for their interest, contribution and creative input to the *Tg*(GFAP-LTa,LTb) mouse model presented in the second chapter; Prof. Burkhard Becher and Laura Codarri for providing us with the 2D2 transgenic mice.
Furthermore, all the colleagues and collaborators who contributed to the success of my thesis: Mirzet Delic, Petra Schwarz, Birgit Riepl, Renaud Maire, Monika Wolf, Timo Böge, Friederike Böhm, Donal McHugh, Tracy O'Connor, Annika Armulik, Mario Hermann, Mario Nuvolone, Tiziana Sonati and Paolo Dametto. Special thanks goes to my new office, Jana Sponarova, Badma Segarane, Uli Hermann, Bei Li and Caihong Zhu for involving me in many interesting scientific and less scientific discussion and contributing to a good working atmosphere.
Moreover, I would like to thank to my friends in an around Zurich and back in Hungary, who supported me in their own ways, made my life easier in the difficult times and made me happier.

Finally, I wish to express my deepest gratitude to my family, especially to my dear parents for the unconditional love and support and for always believing in me. All of this certainly would not have been possible without you setting the example for me.